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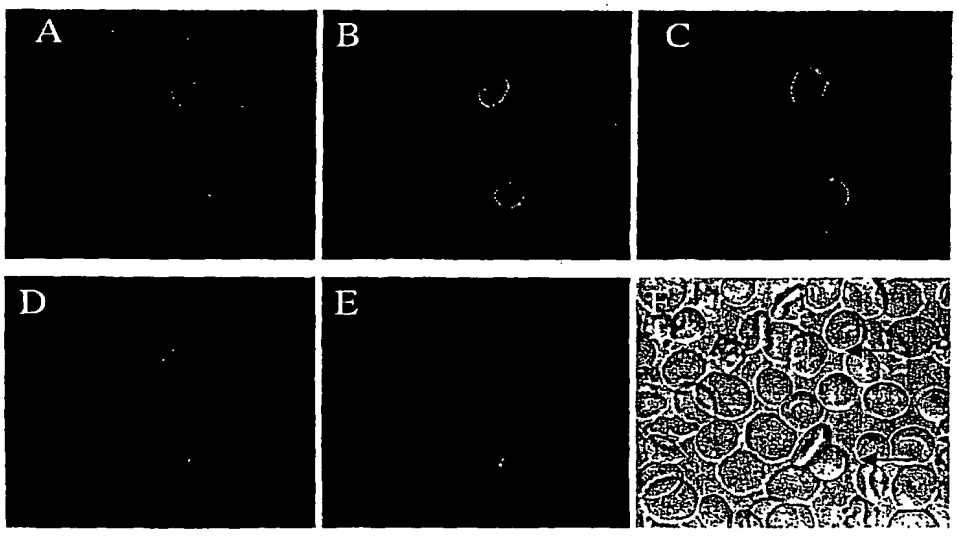

(57) Abstract: This application discloses novel dermaseptin derived peptides with a broad range antimicrobial activity and a reduced hemolytic activity. The affinity of the peptides for the plasma membrane of red blood cells (RBCs), allows the use of RBCs as a drug delivery vehicle.

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MULTI-FUNCTIONAL ANTIMICROBIAL PEPTIDES

FIELD OF THE INVENTION

The present invention relates to peptides derived from dermaseptin S4, and the uses thereof. More specifically, the present invention relates to the antimicrobial activity of these peptides, their ability to bind stably to the membrane of red blood cells, affinity driven transfer of the peptides from the membrane of red blood cells to microbial cells, and methods for using red blood cells with peptides attached to the cell membrane as carriers for drug delivery.

BACKGROUND OF THE INVENTION

Antimicrobial peptides are an essential defense component of both invertebrates and vertebrates, controlling cell proliferation of invading pathogens [1-3]. Antimicrobial peptides display a large heterogeneity in primary and secondary structures, but share common features such as amphipathy and net positive charge, which seem to form the basis for their cytotoxic function. Although their precise mechanism of action is not fully understood, antimicrobial peptides are believed to kill the microbial cell by destabilizing the ordered structure of the cell membrane via either a "barrel stave" mechanism or a non-pore carpet-like mechanism [4-5].

Antimicrobial peptides are potentially active against a large spectrum of microorganisms, yet they are generally non-toxic to normal mammalian cells. The molecular basis for this selectivity is ill defined, but is believed to result from differences in the membrane fluidity and negative charge density in target microbial cells versus non-target cells which result from differences in the lipid composition. A large body of experimental data demonstrates that factors affecting membranolytic properties include the peptide's amphipathy and positive charge distribution [6-12]. Isomers composed of all D amino acids display identical potency as the all L counterparts, which implies that the mechanism of action of their antimicrobial activity is not mediated by interaction with specific receptors, and that peptide-based antibiotics

could escape some of the mechanisms involved in multidrug resistance.

The dermaseptins are a large family of antimicrobial peptides of between 28 to 34 amino acids, expressed in the skin of tree frogs belonging to the genus *Phyllomedusinae* [13-15]. The dermaseptins are linear polycationic peptides, with an amphipathic α-helical structure in apolar solvents [16], and have a cytolytic activity *in vitro* against a broad spectrum of pathogenic microorganisms (bacteria, protozoa, yeast and filamentous fungi). Dermaseptins are also potent killers of non-growing and slow-growing bacteria [19] suggesting a potential use in the eradication of bacteria placed in a dormant state and/or subject to low oxygen tension. By contrast, most conventional bacteriocidal or bacteriostatic antibiotics are not effective against dormant bacteria, or those under conditions of low oxygen. Dermaseptins have also been demonstrated to kill spoilage yeast [20].

Using phospholipid liposomes, and live cells, the selective antimicrobial action of these peptides was demonstrated to be mediated by selective interaction of the amphipathic α-helix moiety with the plasma membrane phospholipids [7, 10, 12, 17, 18, 21] leading to microbial cell permeabilization and killing. Many antimicrobial peptides, including the dermaseptins, are known to bind avidly to the membrane of model liposomes or model cells [1, 7, 10, 12, 17]. For instance, the partition coefficient (K_p) of some dermaseptins in the presence of phospholipids was calculated to be in the range of $10^6 M^{-1}$ [10]. Binding of this class of peptides is believed to represent an early step in a series of events that ultimately leads to a polymerization of the membrane-bound peptide that destabilizes the microbial membrane structure, resulting in cell permeabilization and death.

Among natural dermaseptins, dermaseptin S4 (S4) is highly toxic to erythrocytes, in contrast to other dermaseptins, such as dermaseptin S3 (S3) [12]. Red blood cells were visualized by video microscopy 5 seconds after addition of rhodaminated peptides S3 and S4. Both S3 and S4 was seen at the RBC membrane, however S4 had lysed the RBC and was associated with the 'ghost' membrane. S3 selectively kills the intra-erythrocytic parasite within *Plasmodium falciparum* infected RBCs, unlike S4 that is toxic to both the parasite and its host red blood cell.

In other respects S3 and S4 are very similar; both are highly lipophilic, they bind

with similar affinity to phosphatidyl serine/phosphatidyl choline vesicles, and are equally potent in permeabilizing them. However, NMR and fluorescence methods indicated that S4 is more highly aggregated in aqueous solutions than S3. This led to the proposal that the peptide's aggregation state in solution might be an important factor affecting selective cytotoxicity [12], and suggested that if aggregation is reduced, S4 might become an effective antibacterial agent.

Terminal amino acid deletion from a variety of natural antimicrobial peptides has shown that potency could be maintained to some extent after several C-terminal deletions. The 19 C-terminal residues from the 34 amino acid peptide dermaseptin S1 did not retain the antimicrobial activity of the parent peptide, yet the N-terminal 18 residues did [16]. Similarly, the C-terminal sequence of dermaseptin S3 could be reduced substantially and still conserve large spectrum antimicrobial activity over bacteria, yeast and filamentous fungi [18, 20]. In contrast, deletion of the N-terminal 2 to 4 residues were shown to result in reduced antimicrobial potency of dermaseptin S1 [16], dermaseptin B1 [10] and magainins [22].

There is a clear medical need to identify peptides which have an improved antimicrobial activity. Furthermore, at antimicrobial concentrations, the dermaseptins are essentially non-toxic to erythrocytes, with the exception of dermaseptin S4 which is especially toxic to erythrocytes. Thus there is a clear need to identify peptides, derived from dermaseptin S4, with a reduced hemolytic activity, to increase their potential use as antibiotics *in vivo*.

Systemic administration and delivery of drugs, including peptide drugs, is often challenged by an impressive array of problems. For instance, the drug may be sensitive to degrading or neutralizing blood components which reduce its actual concentration in the blood, or its bioavailability. Similarly, the drug may have difficulty in reaching its target due to its inability to cross barriers, or an inability to expose a given conformation. Failure to solve these problems may thus result in reduced efficacy, toxicity, and a host of undesirable side effects. Various targeting, formulation and delivery systems have been devised to overcome these difficulties, including the use of red blood cells as drug carriers.

The potential use of red blood cells (RBCs) as a carrier system for transport and

delivery of drugs has been well documented. Various methods have been disclosed for encapsulation of drugs within the RBCs, [e.g., 23-24]. Most methods involve the dialysis of RBCs against a hypotonic solution, i.e. causing partial lysis of the cell, the addition of the substance to be encapsulated, and resealing of the lysed RBCs. Molecules encapsulated in RBCs are protected against the metabolism of blood components, and allows the drug to reach its site of action without being broken down or eliminated from the body. Three review articles [25-27] describe methods and practices of using RBCs as carriers for drug delivery. All three articles describe the encapsulation of drugs inside the RBC, but none, however, disclose the attachment of the drug to the RBC membrane.

Some methods for binding drugs and peptides to the red blood cell membrane have been disclosed.

The anticancer drug daunorubicin diffuses rapidly from RBCs, and encapsulation cannot provide a method of delivery of such a drug. Daunorubicin was covalently linked to erythrocyte ghost membranes using glutaraldehyde and cis-aconitic acid linking arms [28].

European Patent No. 0 362 758 to Youssef *et al.* discloses a method of electro-inserting a protein, bearing a hydrophobic membrane spanning sequence, into the membrane of an animal cell such as a red blood cell. The cells are suspended in a buffered solution of the protein, exposed to an electric field, and after insertion, the membranes resealed by contact with a resealing medium.

Published PCT Application WO 98/02454 to R. Smith *et al.* discloses a method of localizing soluble exogenous polypeptides on the surface of cellular membranes, such as the membranes of red blood cells, by complexing the polypeptide with two or more heterologous membrane binding elements. Each of the heterologous membrane binding elements has a low membrane binding affinity, but in combination they provide a high affinity for specific membranes, thus targeting the polypeptide to specific cell types.

There is a clear need in the art for simplified methods for using RBCs as drug delivery carriers in which the drug may be directed to specific target cells, and which overcome the disadvantages in the prior art such as lysis of the RBC, or causing the elimination of the RBCs from the circulation.

It is a primary objective of the present invention to provide new dermaseptin S4 derived peptides with increased antimicrobial activity and a reduced hemolytic activity. It is a further objective of the present invention to use these new and improved dermaseptin-derived peptides in a method of drug delivery through affinity-driven attachment to, and affinity-driven release from, the cell membrane of red blood cells.

SUMMARY OF THE INVENTION

The present invention is directed to novel dermaseptin S4 derived peptides which have an increased antimicrobial activity and a reduced hemolytic activity. These derived peptides include deletion derivatives, substitution derivatives, and combined substitution/deletion derivatives of dermaseptin S4.

The present invention exploits the affinity of certain lipophilic molecules to the plasma membrane of red blood cells (RBCs), which allows the use of RBCs as a transport vehicle to deliver drugs to distant targets. The lipophilic molecule may be peptides, and may be dermaseptin S4 derived peptides. This drug delivery system involves the transient "loading" of RBCs with a non-toxic lipophilic "hook" molecule (e.g., an amphipathic peptide). Such a lipophilic "hook" molecule has enough affinity for the RBC's plasma membrane to bind to the membrane and be transported in the blood circulation, but given the opportunity, the lipophilic "hook" molecule will exit its position and transfer to another (target) cell for which it has a greater affinity. Preferred characteristics of such lipophilic "hook" molecules are that they are non-toxic to the RBC, and non-hemolytic.

If the hook molecule is itself the active drug, a further preferred characteristic is a high intrinsic activity against its intended target, e.g. a high antimicrobial activity. The dermaseptin S4 derivative peptides of the present invention possess these preferred characteristics. The efficacy of such an affinity driven transfer system was demonstrated experimentally by the transfer of antimicrobial peptides from pre-loaded RBCs to bacteria, yeast and protozoan target cells. If a drug molecule is attached to the lipophilic "hook" molecule, the drug molecule and the lipophilic "hook" molecule are transferred together.

The present invention is also directed to methods for delivery of drugs, or other molecules, via the blood circulation of an animal. More specifically, the present invention seeks to provide methods for drug delivery for the treatment of, for example, infectious diseases or cancer. The "hooks" may contain intrinsic activity such as when the hook is an antimicrobial peptide. Alternatively, an antimicrobial hook molecule, or an inactive lipophilic hook molecule, may be coupled to other drugs, in which case, the hook is used to anchor the drug to the RBC.

The present invention is thus also directed to a novel group of dermaseptin S4 derived peptides which may be used for their intrinsic antimicrobial activity, or as lipophilic "hooks" molecules. These peptides bind to the RBC membrane, are substantially non-toxic to the RBC, and furthermore, also possess an effective antimicrobial activity.

The present invention is further directed to dermaseptin S4 derived anti-microbial peptides which, when bound to the RBC membrane, provide protection to the RBC against microbial infection.

There is thus provided in accordance with a preferred embodiment of the present invention an amphipathic dermaseptin S4 derivative peptide selected from the group consisting of Seq. Id Nos. 2-19, and the sequence X-ALWKTLLKKVLKA-Y, where X and Y can be amine, tetramethyl-rhodamine, lissamine-rhodamine, lysine, an amino acid, Fmoc, Boc, a hydrophobic molecule or atom, the peptide having an antimicrobial activity and low hemolytic activity, an analog thereof, and pharmaceutically acceptable salts thereof.

Further in accordance with a preferred embodiment of the present invention there is provided a nucleic acid sequence encoding the peptide.

Still further in accordance with a preferred embodiment of the present invention the hemolytic activity of the peptide has an LC_{50} greater than about 10μM.

Additionally in accordance with a preferred embodiment of the present invention the peptide has substantially no hemolytic activity.

Further in accordance with a preferred embodiment of the present invention the antimicrobial activity of the peptide is a broad range antimicrobial activity.

Still further in accordance with a preferred embodiment of the present invention the broad range antimicrobial activity includes at least one of an antiviral, antibacterial, antiprotozoan, antimycoplasma and antifungal activity.

Additionally in accordance with a preferred embodiment of the present invention the antimicrobial activity is a prophylactic antimicrobial activity.

Further in accordance with a preferred embodiment of the present invention the peptide has a low hydropathic index and a high positive charge.

Still further in accordance with a preferred embodiment of the present invention the low hydropathic index is less than about 10 and the high positive charge is greater than about 4.

Additionally in accordance with a preferred embodiment of the present invention the peptide is in a substantially non-aggregated state in aqueous solution.

Further in accordance with a preferred embodiment of the present invention there is provided a drug molecule attached to the peptide.

Still further in accordance with a preferred embodiment of the present invention there is provided a prophylactic molecule attached to the peptide.

Additionally in accordance with a preferred embodiment of the present invention the drug molecule is an antimicrobial or an anticancer drug.

Also in accordance with a preferred embodiment of the present invention the peptide is effective to activate cells of the monocyte or macrophage lineage or other lymphoid cells.

Further in accordance with a preferred embodiment of the present invention the peptide is suitable for stable attachment to the membrane of a red blood cell (RBC).

Still further in accordance with a preferred embodiment of the present invention peptide the stable attachment does not substantially affect the antimicrobial activity

Additionally in accordance with a preferred embodiment of the present invention the attachment is an affinity-driven attachment.

Also in accordance with a preferred embodiment of the present invention the peptide is suitable for release from the RBC membrane in an affinity-driven release at a region of higher affinity for the peptide than the affinity of the peptide for the RBC membrane.

There is further provided according to another preferred embodiment of the present invention a drug delivery method including affinity-driven attachment of at least one lipophilic "hook" molecule to the cell membrane of at least one red blood cell (RBC), and affinity-driven release of at least one of the at least one lipophilic "hook" molecules from the cell membrane of the at least one RBCs at a region of higher affinity

for the lipophilic "hook" molecule than the affinity of the lipophilic "hook" molecule for the cell membrane of the at least one RBC,

Further in accordance with a preferred embodiment of the present invention the at least one lipophilic "hook" molecule is a drug molecule.

Still further in accordance with a preferred embodiment of the present invention the at least one drug molecule is attached to the at least one lipophilic "hook" molecule.

Additionally in accordance with a preferred embodiment of the present invention the at least one lipophilic "hook" molecule is an amphipathic dermaseptin S4 derivative peptide selected from the group consisting of Seq. Id Nos. 2-19, and the sequence X-ALWKTLLKKVLKA-Y, where X and Y can be amine, tetramethyl-rhodamine, lissamine-rhodamine, lysine, an amino acid, Fmoc, Boc, a hydrophobic molecule or atom, the peptide having an antimicrobial activity and low hemolytic activity, an analog thereof, and pharmaceutically acceptable salts thereof.

Still further in accordance with a preferred embodiment of the present invention the attachment occurs exteriorly to the body.

Additionally in accordance with a preferred embodiment of the present invention the attachment occurs within the bloodstream of a body.

Further in accordance with a preferred embodiment of the present invention the at least one RBCs are introduced into the blood circulation of an individual.

Further in accordance with a preferred embodiment of the present invention the region of higher affinity is a microbial pathogen.

Still further in accordance with a preferred embodiment of the present invention the microbial pathogen is selected from the group consisting of viral, bacterial, protozoan, mycoplasma and fungal pathogens.

Additionally in accordance with a preferred embodiment of the present invention the drug molecule is selected from the group consisting of oxacillin, vancomycin, ceftazidime and imipenem.

Also in accordance with a preferred embodiment of the present invention the region of higher affinity is a cancer or a tumor.

Further in accordance with a preferred embodiment of the present invention the drug molecule is selected from the group consisting of cyclophosphamide, methotrexate, 5-fluorouracil and loeucoverin.

There is also provided according to another preferred embodiment of the present invention a drug delivery method including:

a. attachment of at least one lipophilic "hook" molecule to at least one drug molecule;

b. affinity-driven attachment of at least one of the at least one lipophilic "hook" molecules to the cell membrane of at least one RBC; and

c. affinity release of at least one of said lipophilic "hook" molecules from at least one of the at least one RBCs, at a region of higher affinity for the lipophilic "hook" molecule than the affinity of the lipophilic "hook" molecule for the RBC,

wherein steps a and b can be performed sequentially in either order, or substantially simultaneously.

Further in accordance with a preferred embodiment of the present invention the affinity-driven attachment of step b occurs within the blood circulation of a body.

There is further provided, according to another preferred embodiment of the present invention, a kit for the attachment of at least one lipophilic "hook" molecule to at least one red blood cell (RBC), the kit comprising the at least one lipophilic "hook" molecule, and reagents suitable for attachment of said at least one lipophilic "hook" molecule to the cell membrane of the at least one RBC.

There is also provided, according to another preferred embodiment of the present invention, a kit for the attachment of at least one drug molecule to at least one red blood cell (RBC), the kit including at least one lipophilic "hook" molecule, reagents suitable for attachment of the at least one drug molecule to the at least one lipophilic "hook" molecule, and reagents suitable for attachment of the at least one lipophilic "hook" molecule to the cell membrane of at least one RBC.

Still further in accordance with a preferred embodiment of the present invention the kit also includes the at least one drug molecule.

Further in accordance with a preferred embodiment of the present invention the kit also includes apparatus suitable for separating the RBCs with the at least one lipophilic "hook" molecule attached, from the other reagents.

Additionally in accordance with a preferred embodiment of the present invention the kit also comprises RBCs in a form suitable for the attachment of the at least one lipophilic "hook" molecule.

Also in accordance with a preferred embodiment of the present invention the kit also includes instructions for the proper usage of the kit.

There is further provided, according to another preferred embodiment of the present invention, a method of introducing a molecule into the interior of a mammalian cell not including a RBC, the method including attachment of the molecule to an amphipathic dermaseptin derivative peptide, and co-incubating said cationic amphipathic dermaseptin derivative peptide in the presence of the mammalian cell, whereby the molecule attached to the cationic amphipathic dermaseptin derivative peptide enters the cell.

There is additionally provided, according to another preferred embodiment of the present invention, a drug delivery method including affinity-driven attachment of a peptide to a red blood cell (RBC) membrane, and affinity-driven release of the peptide from the RBC membrane at a region of higher affinity for the peptide than the affinity of the peptide for the RBC membrane, wherein the peptide is a drug.

There is further provided, according to another preferred embodiment of the present invention, a drug delivery method including

a. attachment of a plurality of drug molecules to a plurality of peptides,

b. affinity-driven attachment of at least some of the plurality of peptides to a plurality of RBC membranes, and

c. affinity-driven release of at least some of the peptides from at least some of the RBCs at a region of higher affinity for the peptide than the affinity of the peptide for the RBC membrane,

wherein steps a and b can be performed sequentially in either order, or substantially simultaneously.

There is additionally provided, according to another preferred embodiment of the present invention, a method of attachment of at least one drug molecule to at least one RBC membrane, the method including co-incubating at least one dermaseptin derived peptide in the presence of at least one RBC, whereby the at least one peptide attaches to the RBC membrane in an affinity-driven attachment.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will be understood and appreciated from the following detailed description, taken in conjunction with the drawings in which:

Figure 1 is a group of six photographs (A-F) showing the RBC membrane localization of rhodaminated K_4-S4(1-13)a as analyzed by fluorescence confocal microscopy. Images A-E are a series of optical sections through two intensely labeled RBCs, showing exclusive labeling of the plasma membrane, and image F is the corresponding light transmission image (arrows point to the labeled RBCs).

Figure 2 is a graph showing the temperature-dependence of the binding kinetics of K_4-S4(1-13)a to human RBCs.

Figure 3 is a graph illustrating the peptide concentration of dermaseptin S4 and its derivatives causing 50%hemolysis of RBCs (LC_{50}).

Figure 4 is a graph illustrating the peptide concentration of dermaseptin S4 and its derivatives causing 50% growth inhibition of *E. coli* (IC_{50}).

Figure 5 is a graph illustrating the lowest peptide concentration that causes 100% growth inhibition of *E. coli* after overnight incubation at 37°C of dermaseptin S4 and its derivatives.

Figure 6 is series of six graphs illustrating the dose response profiles for growth inhibition of *E. coli* and hemolytic activity of dermaseptin S4 and substitution derivatives (6A and 6B) dermaseptin S4 and the deletion derivatives (6C and 6D), and dermaseptin S4 and the substitution/deletion derivatives (6E and 6F) after 3h exposure in culture medium at 37°C. Symbols: S4 = star; D_{20}-S4 = empty circle; D_4-S4 = crossed circle; D_4D_{20}-S4 = filled circle; K_{20}-S4 = empty square; K_4-S4 = crossed square; K_4K_{20}-S4 = filled square; S4(1-20) = empty diamond; S4(1-16) = crossed diamond; S4(1-12) = filled diamond; S4(5-16) = right filled diamond; S4(5-28) = left filled diamond; S4(9-28) = up filled diamond; S4(13-28) = down filled diamond; K_4-S4(1-16) = empty triangle pointing up; K_4-S4(1-16)a = filled triangle pointing up; K_4-S4(1-15)a = empty triangle pointing down; K_4-S4(1-13)a = filled triangle pointing down; and K_4-S4(1-10)a = a cross.

Figure 7 is a graph illustrating the lowest peptide concentration that causes

100% growth inhibition after overnight incubation at 37°C of dermaseptin S4 and its derivatives against *Pseudomonas aeroginosa.*

Figure 8 is a graph illustrating the lowest peptide concentration that causes 100% growth inhibition after overnight incubation at 37°C of dermaseptin S4 and its derivatives against *Yersinia kristensenii.*

Figure 9 is a graph illustrating the lowest peptide concentration that causes 100% growth inhibition after overnight incubation at 37°C of dermaseptin S4 and its derivatives against *Cryptococcus neoformans.*

Figure 10 is a graph illustrating the lowest peptide concentration that causes 100% growth inhibition after overnight incubation at 37°C of dermaseptin S4 and its derivatives against *Candida albicans.*

Figure 11 is a graph illustrating the lowest peptide concentration that causes 100% growth inhibition after overnight incubation at 37°C of dermaseptin S4 and its derivatives against *Leishmania major.*

Figure 12 is a graph illustrating the effect of proteolysis on the fluorescence of rhodaminated peptides in PBS. The hatched columns show the initial fluorescence of rhodaminated dermaseptin S4 (Rho-S4) or the substitution derivatives at 0.35μM. The white columns show the final fluorescence, 120min. after addition of proteinase K.

Figure 13A illustrates the relationship between the fluorescence and the concentration of free rhodamine or rhodaminated peptides in PBS; and Figures 13B, C and D show the dose dependent growth inhibition of Gram negative bacteria: *Escherichia coli, Yersinia kristensenii* and *Pseudomonas aeruginosa,* respectively. Symbols: rhodamine = broken line; S4 = star; D_{20}-S4 = crossed circle; D_4-S4 = empty circle; D_4D_{20}-S4 = filled circle; K_{20}-S4 = crossed square; K_4-S4 = empty square; and K_4K_{20}-S4 = filled square.

Figure 14 shows the effect of high peptide concentrations on the ability of K_4K_{20}-S4 to induce cytotoxicity against *E. coli.* (panel A) *Y. kristensenii* (panel B) *P. aeruginosa* (panel C) *L. major* (panel D) and human RBC (panel E).

Figure 15 is a graph showing second order curve fit of rhodaminated peptides in PBS solution showing the dose-response relationships. Symbols: Rho-S4 = star;

Rho-K_4-S4(1-16)a = square; Rho-K_4-S4(1-15)a = circle; and Rho-K_4-S4(1-13)a = triangle.

Figure 16 shows binding of rhodaminated dermaseptin S4 to RBC (panel A) and to *E. coli* (panel B). Symbols: S4 = star; S4(1-12) = triangle; S4(5-16) = empty circle, K4-S4(1-15)a = square; and K4-S4(1-10)a = filled circle.

Figure 17 is a graph showing Affinity Driven Molecular Transfer (ADMT) of the antimicrobial peptide K_4-S4(1-13)a resulting in inhibition of bacterial growth (Symbols: untreated RBC or wash medium = star; preloaded RBC = filled circle), and a photograph showing a microscope field of fluorescent bacteria.

Figure 18 is a group of four photographs (A-D) which illustrate ADMT of the antimicrobial peptide K_4-S4(1-16)a resulting in inhibition of fungal growth. Figure 18A shows normal growth of *C. neoformance* after co-culture with untreated RBC; Figure 18B shows inhibited growth of *C. neoformance* after co-culture with RBC pretreated with K_4-S4(1-16)a; and Figs. 18C and 18D show inhibited growth of *C. neoformance* after co-culture with RBC pretreated with rhodaminated K_4-S4(1-16)a.

Figure 19 is a graph showing ADMT of K_4-S4(1-16)a from RBC-ghosts to *Leishmania major* promastigotes resulting in irreversible inhibition of growth. White columns represent the promastigote count after incubation in presence of K_4-S4(1-16)a; hatched columns represent the promastigote count after wash and re-incubation in abscence of K_4-S4(1-16)a.

Figure 20 are two photographs (A and B) which illustrate ADMT of the rhodaminated peptides K_4-S4(1-13)a and K_4-S4(1-13)a-NLS from the RBC membrane to HeLa cells, as analyzed by fluorescence confocal microscopy; and

Figure 21 is a graph showing that RBC pretreated with K_4-S4(1-13)a are resistant to infection by *Plasmodium falciparum*.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Dermaseptin S4 Derived Peptides

To identify peptides derived from dermaseptin S4 with a reduced hemolytic activity and with an improved antimicrobial activity, and to identify the factors such as charge, hydrophobicity and aggregation which influence these characteristics in solution, substitution, deletion, and combined substitution/deletion peptides derived from dermaseptin S4 were prepared.

As used herein, a 'derived' or a 'derivative' peptide is a peptide, the amino acid sequence of which is based upon its parent peptide, but which has specific, known differences from the parent peptide. The differences may include, by way of example and not limitation, amino acid substitutions, said substitute amino acids may comprise non-naturally occurring amino acid residues; terminal or internal deletions; terminal or internal modifications; and terminal amino acid residue additions.

A peptide 'analog' is a peptide, having more than about 70%, but less than 100% sequence identity with another given peptide. Such analogs may be comprised of non-naturally occurring amino acid residues, including by way of example and not limitation, homoarginine, ornithine and norvaline, as well as naturally occurring amino acid residues. Such analogs may also be composed of one or a plurality of D-amino acid residues, and may contain non-peptide interlinkages between two or more amino acid residues.

A reduced length is another preferred characteristic of antimicrobial peptides, as smaller peptides have certain advantages over larger ones, such as being cheaper to make, being often more specifically selective in their cytotoxicity and being less likely to induce an immune response. The present invention also contemplates shorter versions up to six residues in length as described in Applicant's US Patent Application No. 08/574.701 to Mor *et al.*, hereby incorporated in its entirety by reference.

The general formula X[K_4-S4(1-13)]Y describes preferred embodiments of the peptides of the present invention. In this terminology, the designation of the parent dermseptin, e.g. S4, is central. Following the designation of the parent dermaseptin, the number and position of the amino acids of the parent peptide that are present in the derivative peptide are indicated in parentheses, e.g. amino acids 1-13 of the 28 amino

acids that comprise S4. Preceding the designation of the parent peptide, amino acid substitutions are indicated by the one letter code for the replacement amino acid followed by a number in subscript which indicates the position of the substitution. X and Y represent modifications which may be made at the N- and C-terminus of these peptides and can be tetramethyl-rhodamine, lissamine- rhodamine, lysine or any other amino acid or series of amino acids, amide, Fmoc, Boc, or any other hydrophobic molecule or atom. Modifcations may be made to the peptides as described in US Patent Application No. 08/574,701, *supra*.

As non-limiting examples, derivatives were prepared as described in Example 1 and listed in Table 1. A set of dermseptin S4 substitution derivatives was made where Asp (D) replaced Met (M) in position 4, Asn (N) in position 20 or both positions, and where the same positions were substituted with Lys (K), i.e. six substitution derivatives in total. A set of deletion derivatives of dermaseptin S4 was prepared wherein the primary structure of dermaseptin S4 was sequentially shortened from the N- and/or C-terminal ends. A set of substitution/deletion dermseptin S4 derivatives, composed of substituted shortened versions of dermaseptin S4 was also prepared. As described hereinbelow several dermseptin S4 derivatives were identified as non-limiting examples of peptides that display both enhanced antibacterial potency and enhanced selectivity.

The present invention also contemplates analogs of the peptides listed in Table 1. Preferably, the analog peptides possess at least about 70% sequence identity to the listed derivatives, more preferably at least about 90% sequence identity.

Peptides of the present invention, and compositions comprising the peptides of the present invention, may be made by standard techniques, such as peptide synthesis or recombinant DNA methodologies, as are known to those of skill in the art. Non-limiting examples of methods for preparing peptides and peptide-containing compositions may be found in Applicant's US Patent Application No. 08/574,701, *supra*.

Also contemplated by the present invention are nucleic acid sequences which encode the peptides of the present invention, and the peptide analogs thereof. The multiplicity of nucleic acid sequences that encode the peptides of the present invention, and the peptide analogs thereof, may be easily deduced from the amino acid sequences by one of average skill in the art.

Properties of Dermaseptin S4 Derived Peptides

Cytotoxicitvof Dermaseptin S4 Derived Peptides

As summarized in Table 2, and as described in more detail in the Examples, the dermaseptin S4 derived peptides all possess antibacterial activity as shown by growth inhibition of *E. coli*, and most possess a significantly reduced hemolytic activity.

The peptide concentration causing 50% lysis of RBC (LC_{50}), and the peptide concentration causing 50% growth inhibition of *E. coli* (IC_{50}) were measured. The Relative selectivity index (RSI), i.e. the ratio of LC_{50}/IC_{50} of a given derivative relative to that of dermaseptin S4, was calculated. The RSI is thus a combined measurement of two preferred characteristics of a peptide: high LC_{50} (reduced hemolysis) and low IC_{50} (increased antimicrobial activity). The higher the RSI, the more preferred the peptide. All the peptides, apart from three of the four full-length singly substituted peptides, D_4-S4, D_{20}-S4 and K_{20}-S4, have an increased relative selectivity index (RSI) compared to S4.

The derivative peptides also possess a broad range antimicrobial activity. As summarized in Table 3, and as described in more detail in the Examples, the peptides demonstrate anti-bacterial activity as demonstrated by growth inhibition of the Gram negative bacteria *E. coli, Pseudomonas aeroginosa and Yersinia kristensii;* anti-fungal activity as demonstrated by growth inhibition of the yeast *Cryptococcus neoformance* and *Candida albicans;* and anti protozoan activity as demonstrated by growth inhibition of the protozoan *Leishmania major.* K_4-S4(1-16)a also possesses antibacterial activity against *Acinetobacter baumanii*, as described in Example 12.

In general, the substitution/deletion set of derivatives possess the most effective broad range antimicrobial activity.

Physical Properties of Dermaseptin S4 Derived Peptides

The charge and the hydropathy index of the dermaseptin derived peptides of Table 1 were calculated as described in Example 3 and listed in Table 2.

The aggregational properties of dermaseptin S4 and its derivatives in aqueous solution, was investigated as described in Example 10. This detection is based on the

self-quenching of rhodamine fluorescence when several rhodamine-labeled peptides are in close proximity, i.e., in an aggregated state. An increase in fluorescence occurs when the aggregated rhodamine-labeled peptide is forced to dissociate, such as when the peptide is cleaved by a proteolytic enzyme. At equal concentrations, the rhodaminated peptides displayed clear differences in their fluorescence intensity, especially when compared to Rho(K_4K_{20})S4. Addition of proteinase K caused dequenching (an increase) of fluorescence indicating that the peptides had been aggregated to various extents. After proteinase K cleavage, all the peptides exhibited very similar levels of fluorescence.

To understand the nature of the aggregation state, the dose dependent fluorescence values of the peptides were measured. The linear nature of, for example, Rho(K_4K_{20})S4 Rho(K_4)S4 plots at low μM concentrations, suggests that these two peptides do not change their state in this concentration range. In contrast, the plots of the remaining rhodaminated peptides deviated from linearity, indicating their change in aggregational state at lower concentrations.

The peptides aggregate in aqueous solutions, to different extents. For example, the aggregational state of dermaseptin S4 was not reduced by negative charge substitutions in peptides D_4-S4, D_{20}-S4 or D_4D_{20}-S4. However, positive charge mono-substitution in position 4, K_4-S4, but not in position 20, reduced the peptide's aggregation state. Peptide aggregation is further reduced in di-substituted K_4K_{20}-S4.

Effect of Aggregation

Unlike other natural dermaseptins, where the positive charges are more or less regularly spaced along the peptide sequence, dermaseptin S4 has four positive charges concentrated in the middle, flanked by two hydrophobic stretches. If one (or both) of the extremities is involved in hydrophobic interactions with other peptides, insertion of positive charges might force depolymerisation due to charge repulsion.

Titration of the rhodaminated peptides in PBS showed that the doubly substituted peptide Rho(K_4K_{20})S4 was clearly less aggregated than native dermaseptin S4. Moreover, the fact that of the two corresponding singly substituted peptides, Rho(K_{20})S4 is aggregated but Rho(K_4)S4 is not, further indicates that the N-terminal hydrophobic stretch is primarily responsible for aggregation. Deletion experiment

performed on the sequence of dermaseptin S4, where the hydrophobic stretches were sequentially truncated, confirmed this view. In accordance with this view, the effect of negative charge insertion within a high positive charge environment is likely to be negligible or even to encourage aggregation.

Without being bound by theory, the factors believed to influence cytotoxicity include peptide size, amphipathy and positive charge distribution. The peptides' positive charge is consistent with their affinity for microbial membranes bearing negative charges. The peptides amphipathy is consistent with their capacity to intercalate into membranes. These actions are likely to be facilitated by their small size. A comparison of the data of antimicrobial activity, hemolytic activity and aggregation state of the peptides of the present invention indicates that peptide organization in solution represents another factor. The peptides' aggregation profile correlates very well with their antibacterial potency. Whereas aggregated peptides are weakly active against Gram negative bacteria, the less aggregated peptides are highly active.

It is believed that peptide aggregation in solution effects the cytotoxicity induced by, and hence the selectivity of, antimicrobial peptides. This may be of utmost importance in the design and assessment of potent and/or selective peptide-based antimicrobials. The view that hydrophobic interactions involving primarily the N-terminal sequence of dermaseptin S4 (and to a lesser extent the C-terminus) are responsible for the peptide's aggregation in solution is confirmed by the results obtained with the deletion derivatives. In this respect, dermaseptin S4 presents a surprisingly and unexpectedly different behavior from the other dermseptins since shortening the S4's N-terminal domain by 4 residues actually enhanced antibacterial activity while simultaneously reduced the hemolytic activity. An explanation for this behavior might reside in a particular sequence motif of the N-terminus.

Peptides whose N-terminus is moderately hydrophobic had reduced hemolytic activity, such as in S4(5-28) (whose N-terminus is TLL-), or all the deletion derivatives, as well as all natural dermaseptins (whose N-terminus is ALW-). In contrast, peptides whose N-terminus is highly hydrophobic are hemolytic. Such examples include the native dermaseptin S4 and its derivative S4(1-20) whose N-terminus is ALWMTLL-.

The dermaseptin derivative peptides of the present invention were unexpectedly

found to be particularly susceptible to N-terminal modifications. If the N-terminal sequence is involved in peptide aggregation, its deletion probably reduces aggregation or even produced monomeric peptides. The N terminal deletion peptide S4(5-28) and other deletion derivatives, displayed potent antibacterial activity and weak hemolytic activity. In addition, the fluorescence plots of rhodaminated peptides indicated that the highly antimicrobial and weakly hemolytic peptides K_4-S4(1-15)a and K_4-S4(1-13)a were much less aggregated than dermaseptin S4. In contrast, S4(1-20) is probably still aggregated, which may explain why this peptide displayed strong hemolytic activity.

It was previously shown that the length of various synthetic model polycationic peptides influences their hemolytic properties — shorter peptides display reduced hemolytic activity, accompanied by reduced antimicrobial potency, compared with their longer derivatives [8, 9]. In contrast, the present shortened dermaseptin S4 derivatives display reduced hemolytic activity combined with enhanced antimicrobial activity.

Effect of Charge vs Hydrophobicity

The relative force of two parameters affecting cytotoxicity, i.e., charge and hydrophobicity, was assessed. With respect to the issue of selective toxicity between bacteria and RBC, our data showed that surprisingly and unexpectedly, the hydrophobicity – not the charge –is the critical parameter for selectivity. Comparison of the peptides' hydropathic index (HI) and charge, with their hemolytic and antimicrobial activities supports this view. These properties of dermaseptin S4 and derivatives are listed in Table 2.

By way of illustration, a comparison can be drawn between dermaseptin S4 and the C-terminally deleted derivatives S4(1-20) and S4(1-16), which have an identical net positive charge. Dermaseptin S4 (HI 28.9) has potent hemolytic activity and weak antibacterial activity, S4(1-20) (HI 15.2) has somewhat reduced hemolytic activity but enhanced antibacterial activity and S4(1-16) (HI 11.3) has even less hemolytic activity and further enhanced antibacterial activity. The relative selectivity index (RSI) of the latter peptides rose by a factor of 16 and 33, respectively.

By way of another illustration, comparison can be drawn between the three

derivatives S4(1-16), K_4-S4(1-16) and K_4-S4(1-16)-$_{NH2}$. These peptides are all the same length of 16 amino acids, but they possess increased charge (4, 5 and 6 respectively) and reduced HI (11.3, 5.5 and 5.5 respectively). S4(1-16) has weak hemolytic activity and relatively weak antibacterial activity. Increasing its charge from 4 to 5 by substitution with lysine (K) reduce its hydropathic index from 11.3 to 5.5. This results in a weakened hemolytic activity and an enhanced antibacterial activity; the relative selectivity index rose from 33 to 651. Further increasing the charge of K_4-S4(1-16) from 5 to 6 by amidation, which does not modify the hydropathic index, results in enhancing both activities evenly by 6 fold. Based on these observations, we conclude that while hydrophobicity contributes to antimicrobial vs. hemolytic specificity, charge contributes to potency.

The complex relationships between hydropathic index and positive charge contributions to the activity of dermaseptin S4 may be summarized as follows:

1. A combination of low hydropathic index (about <10) with high positive charge (about >4) is consistent with low hemolytic activity and high antibacterial activity, e.g. K_4-S4(1-16).

2. A combination of high hydropathic index (about >20) and low positive charge is consistent with high hemolytic activity and low antibacterial activity, e.g. D_{20}-S4.

3. A combination of low hydropathic index and low positive charge is consistent with general reduced cytotoxicity, e.g. S4(1-12).

4. A combination of high hydropathic index and high positive charge is consistent with general enhanced cytotoxicity, e.g. K_4K_{20}-S4.

Intermediate properties balance between the mentioned above extremes, taking into consideration that these relationships are highly susceptible to peptide organization in solution. Thus, even though K_{20}-S4 combines a high hydropathic index with a high positive charge, its high aggregation state in solution is probably responsible for obstructing its antibacterial activity. Whereas the aggregation state of D_{20}-S4 does not hamper its hemolytic activity, and the peptide behaves as predicted.

Preferred characteristics of an antimicrobial peptide, such as low hemolytic activity and high antibacterial activity, are conferred, as in 1. above, i.e., by combination

of low hydropathic index with high positive charge. It is thus another feature of a preferred embodiment of the present invention, to provide a method for producing derivative peptides with improved properties for use as an antibiotic. Such a method comprises modifying a peptide so that the derived peptide has an increased antimicrobial activity, and a reduced hemolytic activity, by modifications, such as substitutions or deletions, which reduce the HI and increase the net positive charge.

Uses of Dermaseptin S4 Derived Peptides

The dermaseptin S4 derived peptides possessing antimicrobial activity as described above can be used as antibiotics in the treatment or prevention of microbial infections. A reduced hemolytic activity is preferred when the route of administration of the peptide to a patient is into the blood circulation. Non limiting examples of methods of treatment, routes of administration, and pharmaceutical formulations suitable for use with the peptides of the present invention may be as described in Applicant's US Patent Application No. 08/574,701, *supra*.

Binding of Dermaseptin S4 Derived Peptides to RBC Membranes

The effect of the amphipathic dermaseptin derivative peptides upon RBCs is different to the effect on all other vertebrate cell-types tested. The peptides of the present invention have the surprising an unexpected property of binding to the red blood cell membrane. Upon simple co-incubation, the peptides of the present invention are able to bind to the RBC membrane without destabilizing it, and become integral, stable, non-toxic membrane components. Figure 1 depicts, as an example, the membrane binding of the rhodamine-labeled non-toxic peptide K_4-S4(1-13)a to human RBCs, as described in Example 4, as analyzed by fluorescence confocal microscopy.

Binding of dermaseptin-derived peptides to the RBC membrane is preferably a) spontaneous; b) rapid, i.e. occurs within seconds; c) receptor independent—both all L- and all D-isomers bind identically; and d) essentially irreversible under hydrophilic conditions—peptide displacement is achieved only in presence of detergents (or biological membranes of specific nature).

It is appreciated that the binding of a dermaseptin-derived peptide to a RBC membrane can comprise binding of the peptide to the membrane of an intact live RBC, or to the membrane of a lysed RBC "ghost".

The rate and amount of peptide binding to RBCs is temperature dependent. Figure 2 shows the binding kinetics of K_4-S4(1-13)a to human RBC at 4°C, 25°C, 37°C and 41°C, as determined after extraction of the membrane-bound peptide, (see Example 5). Levels and rates of peptide to RBC binding increased with increasing temperature. Accordingly, the binding of a lipophilic peptide to RBC according the present invention is preferably performed at elevated temperatures. The method of the present invention may be performed at a temperature between 2°C and 41°C, with either ambient room temperature or body temperature (approximately 37°C for humans) particularly preferred. However, it is appreciated that in those instance, e.g. in some clinical settings, when the need to minimize risks associated with infection is greatest, the present invention may be practiced at a lower temperature.

Peptide binding to RBC can be observed both *in vitro* (as shown in Figs 1 and 2) and *in vivo*. As described in Example 6, K_4-S4(1-13)a was injected into SD rats at 10 mg/kg via cannula to the jugular vein. No sign of toxicity was detected, yet the peptide concentration in the serum rapidly dropped and reached undetectable levels at 15 minutes. In contrast, intact peptide could be recovered by extracting red blood cells collected 15 minutes post injection.

Drug Delivery System

The present invention relates to methods for binding a drug molecule to the cell membrane of a red blood cell (RBC).

The present invention further relates to methods for binding a drug molecule to the cell membrane of a red blood cell, circulation of the drug molecule within the body via the blood circulation, and release of the drug from the RBC at the required site.

Definitions

The following terms, when used in this disclosure, have the meanings ascribed to them below.

Lipophilic "hook" molecule — is a molecule which may be a peptide or a modified peptide, that can attach to the lipid membrane of a red blood cell, and that itself may have a further molecule, such as a drug, attached to it, such that the "hook" molecule hooks the drug to the RBC membrane.

Drug — a chemical substance used in the treatment, cure, prevention or diagnosis of disease, or to otherwise enhance physical or mental well-being.

Cell Membrane — the lipid bilayer containing proteins that is the external boundary of the cell.

Affinity — the mutually attractive force by which a molecule, or specific atoms in a molecule, are attracted to another molecule, or to specific atoms in another molecule.

Affinity driven attachment — the process of (non-covalently) attaching a molecule to another molecule driven by the mutually attractive force between the two molecules.

Affinity driven release — the process by which a first molecule, which is attached to a second molecule by affinity driven attachment, leaves the second molecule at a region of higher affinity for the first molecule, than the affinity between the first molecule and the second molecule.

Affinity driven transfer — the process by which a first molecule, which is attached to a second molecule by affinity driven attachment, leaves the second molecule and attaches to a third molecule by affinity driven attachment, wherein the first molecule has a higher affinity for the third molecule than for the second molecule.

Individual — in the context of this invention, an individual can be from any species which has a blood circulation system which includes red blood cells. This includes individuals from the class *Mammalia*, including humans, class *Aves*, class *Reptilia*, and both bony and cartilaginous fish.

Hemolysis — lysis of a red blood cell, which may be due to hypotonic lysis or due to the lytic effects of another molecule on the RBC membrane.

The method of affinity driven attachment is used in the attachment of the drug to the cell membrane of a red blood cell, and affinity driven release is used in the release of the drug from a red blood cell and its transfer to the site of requirement in the body.

It is appreciated that whole blood comprises a mixture of different cell types in varying proportions. The present invention can be applied to whole blood which comprises a mixture of cell types, within which RBCs are the majority cell type. It is further appreciated that the invention is applicable to substantially pure populations of RBCs, which may have been substantially purified from whole blood according to any of the methods known in the art.

According to a first preferred embodiment, the lipophilic "hook" molecule is itself a drug. The lipophilic hook molecule is attached to the RBC cell membrane by affinity-driven attachment. The lipophilic hook molecule is subsequently released from the RBC, and transferred to a site of higher affinity, in an affinity-driven manner. The lipophilic hook molecule preferably attaches to the RBC during simple co-incubation, using such techniques as are known to one skilled in the art. Most preferably the attachment process is kept sterile, using procedures as are well known in the art. According to one aspect of this embodiment, the lipophilic "hook" molecule may be a dermaseptin derivative peptide, or an analog thereof, as described above, and which possesses an intrinsic antimicrobial activity.

According to a second preferred embodiment a drug molecule is chemically attached to a lipophilic "hook" molecule, in a substantially irreversible (e.g. covalent) manner, and is not subject to the processes of affinity driven attachment or release. In this embodiment the lipophilic hook molecule with attached drug molecule, is attached to the cell membrane of an RBC by affinity-driven attachment. The hook molecule with attached drug molecule, is subsequently released from the RBC, and transferred to a site of higher affinity (the site of release), in an affinity-driven manner.

According to this preferred embodiment, the lipophilic hook molecule with attached drug molecule, is released from the RBC at the site of release, for which the combined affinity of the lipophilic hook molecule and of the attached drug molecule is higher than the affinity of the lipophilic hook molecule for the RBC membrane. In this embodiment, either the affinity of the lipophilic hook molecule for the site of release, or of the drug molecule for the site of release, or a combination of the affinity of both the lipophilic hook molecule and of the attached drug molecule for the site of release, is higher than the affinity of the lipophilic hook molecule for the cell membrane.

According to one aspect of this embodiment, the lipophilic "hook" molecule may be a dermaseptin derivative peptide, or an analog thereof, as described above.

According to a third preferred embodiment a drug molecule is attached to a lipophilic "hook" molecule, in a substantially reversible affinity-driven attachment, and the lipophilic "hook" molecule is attached to the RBC cell membrane by affinity-driven attachment. The drug molecule is subsequently released from the lipophilic hook molecule, and transferred to a site of higher affinity, in an affinity-driven manner.

According to this preferred embodiment the drug molecule is released from the lipophilic hook molecule at a site (the site of release) for which the affinity of the drug molecule is higher than the affinity of the drug molecule for the lipophilic hook molecule. It is appreciated, however, that if the affinity of the drug molecule for the site of release is higher than the affinity of the lipophilic hook molecule for the RBC membrane, the lipophilic hook molecule may transfer to the site of release together with the drug molecule. According to one aspect of this embodiment, the lipophilic "hook" molecule may be a dermaseptin derivative peptide, or an analog thereof, as described above.

According to these second and third preferred embodiments, the drug may be attached to the lipophilic hook molecule at the same or different time and at the same or a different location from the attachment of the lipophilic hook molecule to the cell membrane of the RBC. The drug may be attached to the lipophilic hook molecule, either before or after the lipophilic hook molecule is attached to the RBC cell membrane. Alternatively, the drug may be attached to the lipophilic hook molecule at substantially the same time as the lipophhook molecule is attached to the cell membrane of the RBC.

In one aspect of this invention the lipophilic hook molecule is attached to the RBC cell membrane *in vivo*. The lipophilic hook molecule, or the lipophilic hook molecule with attached drug molecule, is injected into the blood stream of an individual, wherein the lipophilic hook molecule attaches to the cell membrane of the circulating RBC

In another aspect of the invention, RBCs with a lipophilic hook molecule, and optionally a further drug molecule, attached as described hereinabove, are introduced to

the blood circulation of an individual. The RBC act as carriers to deliver the drug to sites within the body having a higher affinity for the lipophilic hook molecule as described above.

In a preferred embodiment red blood cells are extracted from individuals of a single species and after the attachment of a lipophilic "hook" molecule, with or without an added drug molecule, the RBC are transfused to another individual of the same species.

Preferably, the RBCs are extracted from a universal blood donor (e.g. group O of the ABO system). More preferably the treated RBCs individuals are returned to another individual of the same ABO blood group. Even more preferably, in humans, the individuals have very similar blood group antigens of at least one of the ABH, Lewis, Ii, P1/P-related, MN, Ss, Gerbich, Rhesus, Kell, Duffy, and Cromer-related blood group antigen systems, according to the criteria used in determining compatibility as are known in the art. Most preferably the treated RBCs are returned to the individual from which they were extracted.

In another embodiment, the present invention may be practiced in conjunction with methods of seroconversion of human blood to that of a universal donor. For example, group B erythrocytes can be enzymatically converted to group O erythrocytes *in vitro* by using an alpha-galactosidase enzyme [29].

Methods for extracting blood, for typing blood, for storing blood, and transfusing blood are routine clinical practices. The present invention may preferably, in a sterile manner as is known to those of skill in the art, be incorporated along with such clinical practices, to provide blood-typed stocks of RBCs with drugs attached. The stocks of blood cells of the present invention are preferably able to be transported from a central site at which attachment of the drug to the RBC is typically performed, to more dispersed sites where the blood cells of the present invention are typically utilized. The utilization may comprise injecting the RBCs directly into a patient with the condition that the drug is designed to treat. The amount of treated blood injected, or otherwise added, to the blood stream of an individual in a therapeutic manner is preferably determined based upon the efficacy of the drug, and the concentration of the drug on the RBCs, in a manner which is known to one in the art.

Manipulations of whole blood, such as dialysis and leukophoresis, which are performed between removal of blood from the body and return to the body of an individual, are known and routinely practiced. Accordingly, the present invention may be practiced in a similar, clinically acceptable manner, whereby blood is removed from an individual, and the drug is attached to the RBC cell membranes before return of the blood to the body.

The RBCs are optionally substantially purified from the blood before attachment of the lipophilic "hook" molecules to the RBC cell membranes. The non-attached lipophilic "hook" molecules, and/or drug molecules, and/or any other reagents required for the attachment to occur, may optionally be removed before return of the RBCs to the body.

According to a preferred embodiment of the present invention, the drug molecule, or the lipophilic hook molecule, or the combination of the drug molecule and the lipophilic hook molecule, do not stimulate an immune response which would otherwise cause the elimination of these RBCs from the circulation by either a humoral or cellular immune response.

According to an alternative preferred embodiment of the present invention, the cellular target of the drug molecule is a cell of the macrophage/ monocyte lineage. In this embodiment the drug molecule, or the lipophilic hook molecule, or the combination of the drug molecule and the lipophilic hook molecule, preferably stimulate an immune response which causes phagocytosis of the RBC by macrophages, thus introducing the drug into the target cell.

Preferably, the attachment of a lipophilic "hook" molecule to the cell membrane of the RBC does not affect the oxygen binding and transport capabilities of a red blood cell, the half-life of the cell in circulation, the overall morphology of the cell, or any other vital physiological function of the RBC.

According to a preferred embodiment of the present invention, the drug is useful/effective in the treatment, cure, prevention or diagnosis of a disease, and the site of higher affinity for the drug, at which the drug is released from the RBC, is the site at which the drug will be most useful or effective in the treatment, cure, prevention or diagnosis of that disease.

In one embodiment the drug is an anti-cancer drug. Solid tumors are often highly vascularized, and the RBCs will perfuse through the tumor. If the cancer to be treated is a leukemia, lymphoma, or other blood cancer, the RBCs will come into contact with the cancer cells in the circulation. The drug preferably has a higher affinity for the cancer cells than it does for its attachment to the RBC cell membrane. At the site of the cancer, or in the presence of cancer cells, the drug leaves the membrane of the RBC, and is transferred to the cancer cells. This process of release and transfer of the drug is affinity-driven.

The type of anti-cancer drug delivered to an individual will be a suitable drug as is known in the art to treat the specific cancer of the individual to whom the drug is delivered. As non limiting examples, cytotoxic or cytostatic chemotherapeutic agents such as cisplatin, doxorubicin, hexamethylamine and VP-16. As further non limiting examples, where the cancer is breast or colon adenocarcinoma, the drug to be delivered for its treatment can be cyclophosphamide, methotroxate, 5-fluorouracil or loeucoverin.

According to another embodiment, the drug attached to RBCs and delivered by the method of this invention is an anti-microbial drug, which is effective in the treatment, cure or prevention of an infectious disease, or any other condition in which microorganisms are implicated. Some microbial infections are blood-borne and the RBCs will come into contact with such microorganisms in the circulation. Other microbial infections are localized and the RBCs pass through or over the site of localized infection. The drug preferably has a higher affinity for the microbial cells than it does for its attachment to the RBC cell membrane. In the presence of the target microorganisms the drug leaves the RBC cell membrane and is transferred to the microorganism. This process of release and transfer of the drug is affinity-driven.

Yet other microbial infections are localized, and the blood circulation does not come into direct contact with the microorganisms, but passes close to the site of infection, for example, infections within the lymph system, or within the alimentary canal, or within organs such as the brain or the lungs. The drug, having a higher affinity for the microbial cells than it does for its attachment to the RBC cell membrane, when passing close to the target microorganism preferably leaves the RBC cell membrane and is transferred to the microorganisms. This process of release and transfer of the drug is

affinity-driven.

The type of microorganism, against which a drug delivered by the method of the present invention may be useful, may be a virus, bacteria, protozoan, fungus, or mycoplasma, or any other microbial pathogen. The anti-microbial drug delivered to an individual will be a suitable drug as is known in the art to treat the specific microbial complaint of the individual to whom the drug is delivered.

As non-limiting examples, where the microorganism is *Staphylococcus aureus*, the drug to be delivered for its treatment can be oxacillin or vancomycin. Where the microorganism is *Pseudomonas aeroginosa*, the drug to be delivered for its treatment can be Ceftazidime or Imipenem. Further non limiting examples of antimicrobial drugs suitable to be employed in the present invention are described in US Patent Application No. 08/574,701.

According to a preferred embodiment of the present invention, the lipophilic "hook" molecule is a peptide derivative of the dermaseptin family of peptides, as described herein. As described above, the dermaseptin derivative peptides are able to interact with RBCs spontaneously and become associated with or adhere closely to the RBC membrane. Such peptides, furthermore are able to transfer from the RBC membrane to a microbial target in an affinity driven manner. The efficacy of Affinity Driven Molecular Transfer (ADMT) was tested using growth inhibition assays. As described below with reference to Examples 12, 13 and 14, K_4-S4(1-13)a was attached to RBCs by simple co-incubation. After washing to remove unbound peptide, the RBC "loaded" with peptide (loaded RBC, LRBC) were co-incubated, with the Gram negative bacteria *Acetinobacter baumanii*, the yeast *C. neoformance*, or the protozoan *L. major*. Transfer of the peptides to the microorganisms, and significant inhibition of microbial growth were observed.

K_4-S4(1-13)a, as a non-limiting example, and other dermaseptin derivatives, represent a typical "hook" in the sense that a variety of molecules can be hooked (anchored) to RBCs when attached to K_4-S4(1-13)a. Drugs maybe attached to the "hook" both by covalent bonding or by adherence. Drugs or other molecules may be attached to the peptides by linkages such as weak interactions (hydrophobic or electrostatic) to any position of the peptides described herein and/or in US patent

application No. 08/574,701. The "hook" interacts with the membrane while the attached drug remains exposed to the exterior milieu. Upon reaching its specific target for which the drug has a higher affinity (e.g., its receptor) the drug will transfer (accompanied or not by the "hook" depending on the mode of attachment) from the carrier membrane to the target molecule.

As a non-limiting illustration, a nuclear localization signal (NLS) was covalently coupled to K4-S4(1-13)a, as described in Example 15, and shown in Figure 20. The NLS is reported to be responsible for directing a variety of intracellular molecules from the cytoplasm to the nucleus by interacting with a specific receptor bound to the nuclear membrane [30]. The rhodaminated peptide, with and without the NLS construct, were delivered from RBCs to mammalian cells such as HeLa cells and fibroblasts. Using confocal microscopy, rhodaminated K_4-S4(1-13)a was observed exclusively in the cytoplasm of these cells while rhodaminated K_4-S4(1-13)a-NLS was observed in the nucleus as well as in the cytoplasm, thus showing the efficacy of the NLS, and illustrating that a molecule attached to the lipophilic "hook" molecule retains its activity. It is thus appreciated that according to another embodiment of the present invention, there is provided peptides for targeting the nucleus of mammalian cells (excluding red blood cells), prepared by coupling a NLS to a peptide of the present invention.

Prophylactic Uses

The present invention also provides for the use of the peptides of the present invention as a prophylactic measure against microorganisms and microbial disease. Pretreatment of RBCs with an antimicrobial peptide as described above prevents the microorganism from infecting the RBC.

As a non limiting example, K_4-S4(1-13)a prevents the malaria parasite *Plasmodium falciparum* from infecting human RBCs. As described in Example 16, RBC pretreated with K_4-S4(1-13)a were mixed with the infected cultures (cell ratio of 1:1) so that the infection rate at the onset of the co-culture is approximately 50%. For control experiments, untreated RBC were used. Figure 21 shows RBC pretreated with K_4-S4(1-13)a are significantly less prone to infection by *P. falciparum*.

The peptide is preferably injected into an individual as a prophylactic, or may be attached to RBC *in vitro* and the RBC-peptide complex subsequently administered to an individual. It is appreciated that the life-span of a RBC in circulation is approximately 3 months, and so protects at least this long. Thus this form of prophylaxis is especially suitable for tourists and travelers in a region susceptible to *P. falciparum* for extended periods.

Molecules which elicit an immune response are often used as vaccines, as is known in the art. Such molecules may be attached to the peptides of the present invention and administered to an individual, either directly by injection to the blood stream, or after prior attachment to RBCs. In this manner vaccination would occur in an analogous manner to that known in the art, however, the stimulus to the immune system would remain present in the circulation for up to three months. This may provide advantages over prior art vaccinations which are more quickly removed from the blood stream.

Peptide Entry into Cells

Surprisingly and unexpectedly, the effect of amphipathic dermaseptin derivatives upon RBCs is different to the effect on all other vertebrate cell-types tested. Upon simple co-incubation, some amphipathic dermaseptin derivatives are able to become stably associated with the RBC membrane without destabilizing it, and become integral, stable, non-toxic membrane components. In contrast, upon simple co-incubation, the amphipathic dermaseptin derivatives pass through the membrane of other vertebrate cells and cell types, and enter the cytoplasm (without specifically targeting the nuclear membrane).

The ability of peptides to cross the membrane of mammalian cells, and the use of this ability to introduce attached membrane-impermeable molecules, has been described in US Patent No. 5,783,662 to Janmey *et al.*, and US Patent No. 5,877,282 to Nadler *et al.* Thus in an alternative preferred embodiment of the present invention, the novel dermaseptin derivative peptides disclosed herein are new and improved peptides for intracellular targeting and delivery of attached membrane-impermeable molecules

Another preferred embodiment of the present invention provides for kits for the attachment of lipophilic "hook" molecule to red blood cells. Such a kit may include lipophilic hook molecules, and reagents suitable for attachment of the lipophilic hook molecules to RBC membranes. RBCs may be provided in the kit, or may be provided by the user of the kit.

In this embodiment the lipophilic hook molecule, may itself be a drug molecule. Alternatively a drug molecule may be attached to the hook molecule. In such a case the drug molecule itself, and/or reagents for the attachment of the drug molecule to the hook molecule, may also be provided in the kit. The kit may also include apparatus suitable for separating the RBCs with attached the lipophilic "hook" molecule, from the other reagents. The kit may also include instructions for the use of the kit.

EXAMPLES

The following Examples include representative examples of aspects of the present invention. The Examples are intended to illustrate, exemplify, and highlight various aspects of the present invention and not to limit its scope in any way or manner. An artisan of ordinary skill in the art will readily appreciate additional aspects and embodiments of the invention.

MATERIALS AND METHODS

The following materials and methods were generally used in the Examples:

Reagents

HMP-linked polyamide/Kieselguhr resin (Pepsin KA), pentafluorophenyl (Pfp), N^{α}-9-Fluorenylmethyloxycarbonyl- amino acids (Fmoc-amino acids), and 3-hydroxy-2,3-dehydro-4-oxo benzotriazine (Dhbt) esters were from Milligen/Bioresearch. All other reagents for peptide synthesis and cell cultures were analytical grade. Buffers were prepared using mQ double-distilled water (Millipore).

Peptides

Peptides were synthesized by the solid phase method, applying Fmoc active ester chemistry as described [14]. After removal of the Fmoc from the N-terminal amino acid, the peptide was cleaved from the resin with a mixture of 85:5:5:5 trifluoroacetic acid : para-cresol : H_2O : thioanisole (10mg resin-bound peptide in 1ml mixture). The trifluoroacetic acid was then evaporated and the peptide precipitated with ether followed by washing with ether (6x). The crude peptides were extracted from the resin with 30% acetonitrile in water, and purified to chromatographic homogeneity in the range of 98% to >99% by reverse-phase HPLC (Alliance-Waters) equipped with an automatic injector, photodiode array UV detector and Millennium integration software. HPLC runs were performed on a semi-preparative C4 column using a linear gradient of acetonitrile in water (1%/minute), both solvents containing 0.1% trifluoroacetic acid. The purified peptides were subjected to amino acid analysis and electrospray mass spectrometry in order to confirm their composition. Peptides were stored as a lyophilized powder at -20°C. Prior to experimentation, fresh solutions were prepared in water, vortexed, sonicated and centrifuged, and diluted in the appropriate medium.

Peptide labeling

Peptide labeling at the N-terminal amino acid with a fluorescent probe was performed by treating 10mg of resin-bound peptide 0.8 ml dimethylformamide (DMF) containing 20% piperidine in an eppendorf test tube, in order to remove the Fmoc protecting group of the N-terminal amino-acid of the linked peptide. The mixture was agitated for 10min. then centrifuged and the supernatant discarded. The resin-bound peptide was rinsed 3 times in DMF before adding 0.3ml solution of Lissamine rhodamine chloride (10mg/ml) in dry DMF containing 7% v/v diisopropylethylamine. After 24h incubation (stirred in dark, RT) the resin-bound peptide was washed thoroughly with DMF (3x) and diethyl ether/dichloromethane (1:1) and dried at 40°C (4h) then the peptide is cleaved from the resin, precipitated with ether, extracted and purified as described above.

Confocal Microscopy

Confocal microscope images of samples of non-fixed cells treated with rhodaminated dermaseptins were taken using an MRC 1024 confocal imaging system (Biorad, UK). The microscope (Axiovert 135M, Zeiss, Germany) is equipped with a 63x objective (Apoplan; NA 1,4). For rhodamine excitation, an Argon ion laser adjusted at 514nm (Em. 580 df 32nm) was used. Z series images were taken with 0.18μm steps between each focal plane.

EXAMPLE 1

Preparation of Dermaseptin S4 Derived Peptides

S4 and the following three sets of S4 derivative peptides were prepared as described above and are listed in Table 1. A set of dermseptin S4 substitution derivatives was made where Asp (D) replaced Met (M) in position 4, Asn (N) in position 20 or both positions, and where the same positions were substituted with Lys (K), i.e. six substitution derivatives in total. A set of deletion derivatives was prepared wherein the primary structure of dermaseptin S4 was sequentially shortened from the N- and/or C-terminal ends. A set of substitution/deletion dermaseptin S4 derivatives, composed of substituted shortened versions of dermaseptin S4 was also prepared.

Table 1: Sequences of dermaseptin S4 and derivatives.

Peptide	Amino acid sequence	Seq. ID No.
S4	ALWMTLLKKVLKAAAKAALNAVLVGANA	1
Substitution Derivatives		
D_{20}-S4	----------D--------	2
D_4-S4	---D----------	3
D_4D_{20}-S4	---D----------D--------	4
K_{20}-S4	----------K--------	5
K_4-S4	---K----------	6
K_4K_{20}-S4	---K----------K--------	7
Deletion Derivatives		
S4(1-20)	----------	8
S4(1-16)	----------	9
S4(1-12)	----------	10
S4(5-16)	----------	11
S4(5-28)	----------	12
S4(9-28)	----------	13
S4(13-28)	----------	14
Substitution/Deletion Derivatives		
K_4-S4(1-16)	---K----------	15
K_4-S4(1-16)a	---K----------$_{NH2}$	16
K_4-S4(1-15)a	---K----------$_{NH2}$	17
K_4-S4(1-13)a	---K---------$_{NH2}$	18
K_4-S4(1-10)a	---K------$_{NH2}$	19
	X---K---------$_{NH2}$	
	X---K---------Y	

A dash '-' indicates that, at the specified position, the peptide contains the identical amino acid to that of dermaseptin S4, listed above.

a = amide. X and Y can be terminal modifications as described hereinabove.

EXAMPLE 2

Erythrocyte Lysis Assays

The hemolytic effect of a peptide on RBCs was monitored by measuring absorbance of the supernatant, reflecting hemoglobin leakage into the medium as described [12]. The absorbance of replicate, treated samples was compared to the absorbance induced by hypotonic hemolysis (considered as 100% hemolysis).

Human blood was rinsed 3 times in PBS (50mM sodium phosphate, 150mM NaCl, pH 7.3) by centrifugation for 1min at 2700g, then $2.5x10^8$ RBC suspended in 50µl PBS, were added to eppendorf tubes containing 200µl of serial two-fold dilutions of the peptide in PBS, PBS alone (for baseline values), or distilled water (for 100% hemolysis). After incubation (3h under agitation, 37°C) samples were centrifuged and the hemolytic activity was assessed as a function of hemoglobin leakage by measuring the absorbance of 200µl supernatant at 405nm. Statistical data is obtained from at least three independent experiments performed in duplicates.

LC_{50} is defined as the lowest peptide concentration that induced 50% lysis of erythrocytes. Incubation of erythrocytes for 3h in the presence of dermaseptin S4 results in hemoglobin release corresponding to an LC_{50} of approximately 1µM. The LC_{50} of S4 and the deletion, substitution, and deletion/substitution derivatives is shown in Table 2 and in Figure 3.

Negative or positively charged mono-substitutions of S4 did not affect the peptide's hemolytic potency. Di-substitutions on the other hand, had a noticeable effect. Negative charges resulted in reduced hemolytic potency (D_4D_{20}-S4 is 4-5 fold less hemolytic) and positive charges resulted in increased hemolytic potency (K_4K_{20}-S4 is 2 fold more hemolytic). 100% RBC lysis is obtained at a peptide concentration of 3µM.

As shown, deletion of up to 8 C-terminal residues, i.e. S4(1-20), reduces the peptide's hemolytic potency by four-fold (LC_{50} 5µM). Further deletions leads to gradual loss of hemolytic activity, i.e., S4(1-16) and S4(1-12) were respectively 16 and 71-fold less hemolytic than dermaseptin S4.

Stepwise N-terminal deletions leads to progressively less hemolytic peptides.

Thus, S4(5-28), S4(9-28) and S4(13-28) were respectively 12, 17 and 29 fold less hemolytic than dermaseptin S4. Finally, S4(5-16) produced virtually no hemolysis up to 80μM.

All the substitution/deletion derivatives had a weaker hemolytic activity compared to dermaseptin S4 (Table 2), even though their antibacterial activity is equal or enhanced. K_4-S4(1-10)a displays no hemolytic activity up to 100μM.

EXAMPLE 3

Hydropathic index calculation

The peptides' hydropathic index was calculated according to Kyte-Doolittle scale [31] making use of a hydrophobic scale containing hydrophobicity parameters for all 20 amino acids. The scale represents the estimated free energy of transfer of amino acids from an aqueous environment to a hydrophobic environment.

Table 2: Properties of dermaseptin S4 and derivatives.

Peptide	Charge	HI	LC_{50}(μM)	IC_{50}(μM)	RSI
S4	4	28.9	1.4±0.2	40±5	1
Substitution Derivatives					
D_{20}-S4	3	28.9	1.2±0.1	75±6	0.5
D_4-S4	3	23.5	2.3±03	69±11	1
D_4D_{20}-S4	2	23.5	5±1	80	1.5
K_{20}-S4	5	28.5	1.2±0.4	43±8	0.8
K_4-S4	5	23.1	2±0.1	1±0.5	57
K_4K_{20}-S4	6	22.7	0.5±0.1	0.3±0.1	48
Deletion Derivatives					
S4(1-20)	4	15.2	5±1	9±1	16
S4(1-16)	4	11.3	23±1	20±1	33
S4(1-12)	3	9.8	100	53±0.5	54
S4(5-16)	4	4.7	>80	>80	29
S4(5-28)	4	22.3	16.5±0.5	3.5±0.5	135
S4(9-28)	3	19.3	24±1	>80	<9
S4(13-28)	1	19.1	40	>80	<14
Substitution and Deletion Derivatives					
K_4-S4(1-16)	5	5.5	57±3	2.5±0.5	651
K4-S4(1-16)a	6	5.5	10±0.5	0.43±0.1	664
K_4-S4(1-15)a	5	9.4	20±1	0.38±0.1	1503
K_4-S4(1-13)a	5	5.8	57±3	1.35±0.1	1206
K_4-S4(1-10)a	4	4.1	>100	40±1	>71

HI = Hydropathic index, using the Kyte-Doolittle scale [23].

LC_{50} = Peptide concentration causing 50% lysis of RBC (Mean±SD).

IC_{50} = Peptide concentration causing 50% growth inhibition of *E. coli* (Mean±SD).

RSI = Relative selectivity index, the ratio of LC_{50}/IC_{50} of a given derivative relative to that of dermaseptin S4.

BINDING OF DERMASEPTIN S4 DERIVATIVES TO CELL MEMBRANES

EXAMPLE 4

To illustrate peptide binding to the RBC membrane, 50µl of human RBC diluted to 1% in PBS were exposed to rhodaminated K_4-S4(1-13)a in 250µl PBS (1µM, 15min., RT), washed three times by successive centrifugation (2min., 200g) and observed unfixed, and images taken within 5-10 minutes. Rhodamine was excited using an Argon ion laser adjusted at 514nm (Em. 580 df 32nm). A series of optical sections were taken through two intensely labeled RBCs, showing exclusive labeling of the cell membrane, but no labeling in the interior of the cell, and is shown in Figure 1.

EXAMPLE 5

The temperature dependence of the binding kinetics of K_4-S4(1-13)a to RBC is assessed as follows. Human RBC (2.5µl packed cells in duplicates) were exposed to K_4-S4(1-13)a (20µg/ml in PBS) at various temperatures. After the specified incubation period, cells were thoroughly washed (3x) in PBS by successive centrifugation (2 min., 200g) then the pellet hemolyzed in 1ml H_2O. After washing, the bound peptide is extracted from the resulting ghosts in 0.2ml octyl glucoside (1%, 2min., RT) and analyzed by reverse-phase HPLC (Alliance-Waters) equipped with an automatic injector, photodiode array (190-800nm) detector and Millennium integration software. HPLC runs were performed on a semi-preparative C4 column using a linear gradient of acetonitrile in water (1%/minute), both solvents containing 0.1% trifluoroacetic acid. The extracted peptide is identified by electrospray mass spectrometry analysis, and the amount of bound peptide in the extract determined by comparison of the integrated peak area with a calibrated standard curve (220nm). The rate and amount of peptide binding to RBCs was shown to be temperature dependent. Levels and rates of peptide-to-RBC binding increased with increasing temperature. Figure 2 shows the binding kinetics of K_4-S4(1-13)a to human RBC at 4°C, 25°C, 37°C and 41°C, as determined after extraction of the membrane-bound peptide.

EXAMPLE 6

To illustrate peptide binding to the RBC membrane *in vivo*, K_4-S4(1-13)a is injected into SD rats at 10 mg/kg via cannula to the jugular vein. No sign of toxicity is detected, and the peptide concentration in the serum rapidly dropped and reached undetectable levels at 15 minutes. In contrast, intact peptide could be recovered (about 70±20 µg/ml packed cells) by extracting blood cells collected 15 minutes post injection (estimation of the bound peptide is performed as described for *in vitro* experimentsin Example 2 (i.e., hemolysis followed by extraction and analysis by HPLC).

CHARACTERIZATION OF DERMASEPTIN S4 DERIVATIVES

EXAMPLE 7

The antibacterial activity of the dermaseptin S4 derivative peptides was assessed against *Escherichia coli* (TG1), *Yersinia kristensenii* (ATCC 33639) and *Pseudomonas aeroginosa* (ATCC 27853) cultured in 2xTY medium (16g/l trypton, 10g/l yeast extract, 5g/l NaCl, pH 7.4). The peptide's effect was monitored by measuring absorbance at 620nm (i.e. a measure of bacterial growth) and comparing it to the absorbance of control cultures grown in the absence of the peptide.

100µl of bacterial suspension of 10^6 bacteria/ml was added to 100µl of culture medium containing no peptide or different peptide concentrations (serial 2 fold dilutions) in 96 well plates (Nunc). Inhibition of proliferation is determined by optical density measurements (620nm) after the incubation period (3h, 16h and 16h respectively for *E. coli*, *Y. kristensenii* and *P. aeruginosa*) at 37°. Reversibility of bacterial growth inhibition i.e. determination whether the peptide is bacteriocidal or bacteriostatic, was assessed by thoroughly washing the bacteria and re-incubation in fresh peptide-free culture medium.

The concentration of dermaseptin S4 and its derivative peptides that caused 50% growth inhibition of *E. coli* (IC_{50}) is illustrated in Figure 4 and listed in Table 2.

The lowest concentration of dermaseptin S4 and its derivative peptides that causes 100% growth inhibition of *E. coli* after overnight incubation at 37°C (MIC) is illustrated in Figure 5 and listed in Table 3.

Substitution derivatives: Dermaseptin S4 is a weak inhibitor of growth of *E. coli*, barely achieving 50% inhibition at 40μM. The negatively charged mono and di-substitutions further weakened the peptide's antibacterial activity. However, the positively charged substituted peptides K_4-S4 and K_4K_{20}-S4 displayed potent antibacterial activity, 40 and 100 fold more potent than the native peptide respectively. K_{20}-S4 however, is only as active as dermaseptin S4.

After thorough washing and overnight re-incubation in fresh peptide-free culture medium, absorbance measurements confirmed that the growth inhibition is irreversible, i.e. that the peptides are bacteriocidal. Thus, the minimal inhibitory concentrations (0% growth) observed for K_4-S4 and K_4K_{20}-S4 reflect 100% bacterial killing. The remaining substitution derivative peptides, were not able to produce 100% bacterial killing up to 100μM.

Deletion derivatives: Stepwise deletion of the C-terminal 4-12 residues enhanced antibacterial activity. The 16-mer derivative S4(1-16) is twice as active and the 20-mer derivative S4(1-20) is 4-fold more active than the native peptide S4. Moreover, unlike S4 these derivatives were able to induce 100% killing (MIC 30μM and 12μM, respectively). Further C-terminal deletions had the effect of reducing activity, the IC_{50} of S4(1-12) reached 53μM and the MIC reached at 70μM. Surprisingly, deletion of the first 4 N-terminal residues, yielded a peptide S4(5-28) that is 11-fold more active than dermaseptin S4 (IC_{50} 3.5μM). Further deletions however, yielded inactive derivatives, IC_{50} of S4(9-28) and S4(13-28) were not reached at 80μM. Simultaneous deletion of both hydrophobic domains from S4, resulting in peptide S_4(5-16), also resulted in an inactive peptide (IC_{50} 80μM).

Substitution/deletion derivatives: Substitution of the methionine with lysine in position 4 of dermaseptin S4(1-16) enhanced antibacterial activity, as K_4-S4(1-16) is 8 fold more potent than its counterpart and 16 fold more potent than native dermaseptin S4). Moreover, neutralization of the negative charge at the COOH terminus, by conversion of the carboxylate to a carboxamide, yielded a peptide derivative that displayed further increase in potency; K_4-S4(1-16)a is approximately 6 times more potent than K_4-S4(1-16). Stepwise elimination of the C-terminal residues starting from K_4-S4(1-16)a resulted in: a 15-mer derivative with similar antimicrobial potency (IC_{50}

0.38μM), a 13-mer derivative K_4-S4(1-13)a, slightly less potent (IC_{50} 1.35μM) and a 10-mer derivative K_4-S4(1-10)a displaying considerably less antibacterial activity (IC_{50} 40μM). Bacteriocidal activity was verified by washing the samples thoroughly and re-incubating them in fresh peptide-free culture medium. Absorbance measurements performed after overnight incubation confirmed that growth inhibition is irreversibleand that the peptide is bacteriocidal, for all samples displaying 100% growth inhibition (MIC) in the initial experiment. All the peptides tested were bacteriocidal except the native peptide and 4 of the derivatives: S4(1-12), S4(5-16), S4(9-28) and S4(13-28). In addition, the isomer versions of K_4-S4(1-15)a, K_4-S4(1-13)a and K_4-S4(1-10)a composed with all D-amino acids yielded similar results as the all L counterparts, indicating that the observed activity of these peptides is not a function of *E. coli* proteolytic enzymes in particular, or enzymatic activity in general.

The dose response profiles for the growth inhibition of *E. coli* and the hemolytic activity of dermaseptin S4 after 3h exposure in culture medium at 37°C, are illustrated in Figure 6. The dose response profiles for S4 and the substitution derivatives are shown in 6A and 6B; dermaseptin S4 and the deletion derivatives in 6C and 6D; and those for dermaseptin S4 and the substitution/deletion derivatives shown in 6E and 6F.

The lowest concentrations of dermaseptin S4 and its derivatives that cause 100% growth inhibition of *Pseudomonas aeroginosa* after overnight incubation at 37°C (MIC) is illustrated in Figure 7, and this data is listed in Table 3.

The lowest concentrations of dermaseptin S4 and its derivatives that cause 100% growth inhibition of *Yersinia kristensenii* after overnight incubation at 37°C (MIC) is illustrated in Figure 8, and this data is listed in Table 3.

EXAMPLE 8

The anti-fungal activity of the dermaseptin S4 derivative peptides was demonstrated against *Cryptococcus neoformans* and *Candida albicans* essentially as described above for the antibacterial assays except that the culture medioum used was RPMI supplemented with 150mM 3-[n-morpholino]propanesulfonic acid (MOPS), and incubation was performed at 30°C.

The lowest concentrations of dermaseptin S4 and its derivatives that cause 100% growth inhibition of *Cryptococcus neoformans* after overnight incubation at 30°C (MIC) is illustrated in Figure 9, and this data is listed in Table 3.

The lowest concentrations of dermaseptin S4 and its derivatives that cause 100% growth inhibition of *Candida albicans* after overnight incubation at 30°C (MIC) is illustrated in Figure 10, and this data is listed in Table 3.

EXAMPLE 9

Antiprotozoan activity against the promastigote form of *Leishmania major* was demonstrated by counting the number of motile promastigotes in each dermaseptin-treated sample and comparing it to that of samples cultured in peptide-free medium.

The inhibition of proliferation assay was performed by adding $2x10^5$ *L. major* promastigotes (100µl) to 100µl of culture medium (RPMI 1640 complemented with 20% fetal calf serum, 1% penicillin and 1% streptomycin) in 96 well plates (Nunc) containing zero or various peptide concentrations (serial 2-fold dilutions). After the incubation period (3h, 26°C) the number of cells is determined by counting an aliquot from each well on a Neubauer cell counter under a microscope (Olympus IX70).

Protozoacidal was determined by washing the samples (3 centrifugation cycles, 15min., 5,000g), resuspending the pellets and *de novo* incubation overnight in peptide-free fresh medium.

The lowest concentrations of dermaseptin S4 and its derivatives that cause 100% growth inhibition of *Leishmania major* after overnight incubation at 26°C (MIC) is illustrated in Figure 11, and this data is listed in Table 3.

The antiprotozoan activity of dermaseptin S4 and its derivatives parallels their hemolytic activity. Dermaseptin S4 is potent in inducing lysis of *L. major* promastigotes (LC_{50} 2µM), D_4D_{20}-S4 is 5 fold less potent and K_4K_{20}-S4 is 2 fold more potent than the native peptide. Mono-substitutions of either positive or negative charges, did not dramatically affect the anti-*Leishmanial* activity.

Table 3: Properties of dermaseptin S4 and derivatives

Peptide	MIC (μM)					
	Pa	Yk	Ec	Lm	Cn	Ca
S4	>100	>100	>100	1.1	4.4	8.8
Substitution Derivatives						
D_{20}-S4	>100	>100	>100	2.2	4.4	35
D_4-S4	>100	>100	>100	1.1	5	35
D_4D_{20}-S4	>100	>100	>100	2.2	17.5	>100
K_{20}-S4	>100	>100	>100	4.4	4.4	17.5
K_4-S4	4.4	2.2	2.2	1.1	2.2	35
K_4K_{20}-S4	4.4	1.1	1.1	1.1	2.2	2
Deletion Derivatives						
S4(1-20)	25	25	12	25	3.6	15
S4(1-16)	100	100	28	50	8.8	17.5
S4(1-12)	>100	>100	>100	>100	35	>100
S4(5-16)	>100	>100	>100	>100	>100	>100
S4(5-28)	>100	25	5.3	12.5	7	>100
S4(9-28)	100	>100	>100	>100	>100	>100
S4(13-28)	>100	>100	>100	>100	>100	>100
Substitution and Deletion Derivatives						
K_4-S4(1-16)	25	25	3.5	50	8.8	35
K_4-S4(1-16)a	6	6	0.9	6	2.2	8
K_4-S4(1-15)a	12	8	0.9	12.5	2.2	8
K_4-S4(1-13)a	25	25	2	25	4.4	17
K_4-S4(1-10)a	>100	>100	>100	>100	35	35

MIC = Minimal Inhibitory Concentration, the lowest peptide concentration that causes 100% growth inhibition after overnight incubation at 37°C.

Pa = *Pseudomonas aeroginosa*; Yk = *Yersinia kristensenii*; Ec = *Escherichia coli*; Lm = *Leishmania major*; Cn = *Cryptococcus neoformans*; Ca = *Candida albicans*.

EXAMPLE 10

Aggregation in Solution

The aggregational properties of dermaseptin S4 and its derivatives in aqueous solution, were determined using rhodamine-labeled peptides as described [12]. This detection is based on the self-quenching of rhodamine fluorescence when several rhodamine-labeled peptides are in close proximity, i.e., in an aggregated state. An increase in fluorescence should occur when the aggregated rhodamine-labeled peptide is forced to dissociate, such as when the peptide is cleaved by a proteolytic enzyme.

Titration and proteolysis of rhodaminated peptides

1µl of free rhodamine or rhodaminated peptides stock solutions (1mg/ml in DMSO) were successively added to 1ml PBS and the fluorescence recorded (excitation 559nm, emission 583nm) on a Perkin-Elmer fluorimeter. Disruption of the self-quenched fluorescence of the polymers was obtained by measuring the fluorescence 120min. after addition of 1 unit proteinase K (Sigma) to 0.35µM of each peptide.

Alternatively, the rhodaminated peptides were titrated in PBS onto 96 well plates (final volume 200µl, starting from stock solutions in DMSO. Fluorescence (Ex.535nm, Em.590nm) is measured using a Spectrafluor Plus apparatus (TECAN).

The results of the proteolytic experiments are shown in Figure 12. The hatched columns represent the fluorescence at 583nm (characteristic of rhodamine emission peak) of the rhodaminated peptides (0.35 µM) in solution, before proteolytic cleavage. At equal concentrations, the rhodaminated peptides displayed clear differences in their fluorescence intensity, especially when compared to Rho(K_4K_{20})S4. Addition of proteinase K caused dequenching (an increase) of fluorescence indicating that the peptides had been aggregated to various extents. The white columns show the final fluorescence, 120min. after addition of proteinase K. After proteinase K cleavage, all the peptides exhibited very similar levels of fluorescence.

To understand the nature of the aggregation state, the dose dependent fluorescence values of the peptides were measured. Panel A of Figure 13 depicts a typical plot of fluorescence versus concentration of rhodaminated peptides. The linear nature of the Rho(K_4K_{20})S4 and Rho(K_4)S4 plots, at low µM concentrations, suggests that these two peptides do not change their aggregation levels in this concentration

range. In contrast, the plots of the remaining rhodaminated peptides deviated from linearity, indicating their change in aggregational state at lower concentrations.

The peptides aggregate in aqueous solutions, to different extents. The aggregational state of dermaseptin S4 is not reduced by negative charge substitutions (D_4-S4, D_{20}-S4 or D_4D_{20}-S4). However, positive charge mono-substitution in position 4, but not in position 20, reduced the peptide's aggregation state. Peptide aggregation is further reduced in di-substituted K_4K_{20}-S4.

Rhodamination itself was found not to affect the peptides' cytotoxic properties, as previously observed for other dermseptins [7. 10, 12]. Rhodaminated S4, K_{20}-S4 and K_4K_{20}-S4 were examined in parallel to the non-labeled versions and no significant difference was observed in their antiprotozoan or hemolytic potency.

The order of aggregation states displayed by the rhodaminated peptides correlated nicely with antibacterial potency of dermaseptin S4 and its derivatives (Figure 13, Panels B). Whereas all aggregated derivatives (D_4-S4, D_{20}-S4, D_4D_{20}-S4 and K_{20}-S4) were weakly active against *E. coli*, the least aggregated peptide (K_4K_{20}-S4) is the most active. K_4-S4 displayed an intermediate behavior. The same correlation is seen against other Gram negative bacteria such as *Y. kristensenii* and *P. aeruginosa* (Figure 13, Panels C and D, respectively).

The di-amino acid substitution of the sequence of S4 to become K_4K_{20}-S4, which increases the peptide'net positive charge, considerably affects its tendency to form aggregates in aqueous solution. These results suggest that K_4K_{20}-S4 might aggregate at high concentrations.

To verify that possibility and assess its implications, the cytotoxicity of K_4K_{20}-S4 is investigated at some 100-fold higher concentration (stock solution of 1mM). At 200μM, K_4K_{20}-S4 was devoid of activity against *E. coli* (Figure 6, panel A). Gradual recovery of activity is obtained by performing serial 2-fold dilutions in the culture medium, and activity is fully recovered at around 10-20μM. Lower peptide concentrations are less potent: IC_{50} is increased 2-3 fold with stock solutions of 10μM.

Reduced cytotoxicity at high peptide concentrations, with higher activity regained after dilution, is observed against other Gram negative bacteria (*Y. kristensenii* and *P. aeruginosa*. At the highest concentration tested (150μM) the peptide lost almost

50% of its potency against either bacteria (Figure 14, panels B and C, respectively). In contrast, the antiprotozoan activity and the hemolytic activity of K_4K_{20}-S4 were not reduced at high peptide concentrations (Figure 14, panels D and E, respectively).

The aggregational properties of three short derivatives (K_4-S4(1-16)a, K_4-S4(1-15)a and K_4-S4(1-13)a) were investigated as described above. Figure 15 depicts the fluorescence plots versus the concentration of the rhodaminated peptides. The shape of the plots suggests that unlike dermaseptin S4, the deletion derivatives did not change their aggregation state as concentration increased, indicating that the deleted amino acids comprise at least part of the amino acid sequence involved in aggregation.

EXAMPLE 11

Binding experiments

To assess binding of various dermaseptins to RBC or *E. coli*, the supernatants of preparations used for the bioassays were analyzed in HPLC as described [12], but with the following variation. Cells were added to eppendorf tubes containing various peptide concentrations in the appropriate culture medium (total volume 250µl). After the incubation period, samples were centrifuged and 100µl of the supernatant is analyzed by HPLC as detailed above. Peptide identification is based on retention time and spectral analysis. The amount of peptide present in the supernatant (free peptide) is calculated using standard curves of known concentrations for each peptide.

Additionally, binding of the rhodaminated peptides was assessed by measuring the fluorescence intensity of the supernatant (Ex: 535nm, Em: 590nm) compared to a standard calibration curves using a *Spectrafluor Plus* microplate reader (TECAN, Austria), and the data was processed with the *DeltaSoft 3* analysis program (Princeton, USA).

Figure 16 (panel A) depicts the binding profile of dermaseptin S4, S4(1-12), S4(5-16), K_4-S4(1-15)a and K_4-S4(1-10)a to RBC, based on HPLC analysis. Binding based on the fluorescent peptides is strictly similar.. The plots reveal that dermaseptin binding is practically linear over a wide range of concentrations. At a peptide concentration of 5µM (1 nmol), 100% of S4 is bound to the cell. Observation of the

cells treated with this concentration, showed that no characteristic shape of RBC can be found, instead, only ghosts were present. Yet, incubation of RBC with more than 10 fold higher peptide concentrations resulted in >95% binding. The weakly hemolytic derivative K_4-S4(1-15)a bound to a lesser extent, while the inactive derivative K_4-S4(1-10)a bound the least. In contrast, the short derivatives S4(1-12) and S4(5-16) that had virtually no hemolytic activity were found to bind RBC to a considerable extent.

Figure 16 (panel B) depicts the binding profile of the same peptides to *E. coli*. There also, binding did not always correlate with activity. For instance, 100% of K_4-S4(1-15)a is bound to bacteria at 5μM (1 nmol). Even though all bacteria are killed at this concentration, higher binding of K_4-S4(1-15)a is observed with increasing peptide concentrations. The less active derivative K_4-S4(1-10)a bound less. In contrast, the weakly active dermaseptin S4 and S4(1-12) or the inactive derivative S4(5-16) bound between 80 to 100% over a wide range of concentrations. S4(1-20), S4(1-16) and K_4-S4(1-16)a had similar binding as S4(1-12), K_4-S4(1-13)a had similar binding as K_4-S4(1-15)a, while S4(5-28) had similar binding as K_4-S4(1-10)a (not shown).

The relationship between antibacterial potency and the peptides affinity to bacteria is investigated by analysis of the *E. coli* culture supernatants after the specified incubation period with dermaseptin S4 and all the first generation derivatives. At sample concentration of 70μM, about 90% of S4 (12.6±0.8 nmoles) is estimated to be bound to *E. coli*. All the derivatives, whether they displayed higher or lower activity, were also estimated to bind between 11.9 and 12.6 nmoles. An isomer version of K_4K_{20}-S4 composed with all D-amino acids yielded the same results as the L version with respect to binding, hemolysis and antibacterial activity. Likewise, the all D-amino acids versions of K_4-S4(1-15)a and K_4-S4(1-10)a yielded similar results as the all L counterparts, with respect to their binding to RBC and bacteria, suggesting that the observed properties were not biased by differential susceptibility to proteolytic enzymes (not shown).

AFFINITY DRIVEN MOLECULAR TRANSFER

The efficacy of the principle of Affinity Driven Molecular Transfer (ADMT) is

tested using growth inhibition assays. The experiment involved anchoring K_4-S4(1-16)a, a peptide from the dermaseptin family whose direct antimicrobial activity has been described hereinabove.

Peptide anchoring to RBC is spontaneous and takes place rapidly by simple incubation of RBC in the presence of the peptide for a few minutes. Following anchoring, the RBCs are rinsed to separate loaded RBC (LRBC) from unbound peptide. The LRBCs are co-cultured with the target cell. If the peptide has higher affinity for the target cell, than the RBC, it will transfer from the LRBC to the recipient cell and act upon it. Such transfer can be assessed directly by visualization of the interaction of labeled peptide with the target cell, and indirectly by measuring the induced antimicrobial effect.

EXAMPLE 12

Inhibition of bacterial growth:

Human RBC (1% hematocrit) were washed by repeated (3 times) centrifugations in saline (2min. at 200 x g). The washed RBC were resuspended in 1ml saline, from which 50µl was added to 950µl If K_4-S4(1-13)a in saline. Various concentrations of K_4-S4(1-13)a were used; the highest concentration of K_4-S4(1-13)a being 200µM, and this concentration was diluted by serial twofold dilutions. After 15min. incubation at room temperature the treated RBC were submitted to repeated (3 times) centrifugations in saline (2min. at 200 x g). The supernatants were discarded except for that of the last wash which was used as a control for the presence/ absence of unbound K_4-S4(1-13)a. The pellets containing K_4-S4(1-13)a loaded RBC (LRBC) were suspended in 900µl of 2xTY culture medium, (16g/l trypton, 10g/l yeast extract, 5g/l NaCl, pH 7.4).

To assess the antibacterial effect induced by the ADMT of K_4-S4(1-13)a, the LRBC were co-cultured with the Gram negative bacteria *Acinetobacter baumanii* in 2xTY medium. Inocula of 10^6 bacteria/ml were used, estimated by optical density (O.D.) measurements at 620nm with reference to a standard calibration curve. 100µl of bacterial suspension is added to the 900µl of LRBC and incubated overnight at 37°C under agitation. After incubation, the RBC are separated from the bacteria using a 2µm

filter.

Inhibition of bacterial proliferation is determined by O.D. measurements of the filtrate at 620nm. Control experiments include the use of bacteria co-cultured with RBC that were pre-treated with either K_4-S4(1-13)a-free saline, or with the supernatant from the last wash of K_4-S4(1-13)a-treated RBC.

Bacterial growth is inhibited in a dose-dependent manner, and shown in Figure 17A. Specifically, bacterial growth is completely inhibited in co-cultures in which RBC were pretreated with a K_4-S4(1-13)a concentration of 50μM or higher. No inhibition of bacterial growth is seen in the control experiments. This demonstrates that RBC-bound K_4-S4(1-13)a can inhibit bacterial growth.

To prove that K_4-S4(1-13)a transferred from the LRBC to the bacteria, and to exclude the possibility that the growth inhibition was mediated by RBC-bound K_4-S4(1-13)a, the experiment was repeated using K_4-S4(1-13)a rhodaminated at its amino-terminus (Rho-K_4-S4(1-13)a), and transfer of Rho-K_4-S4(1-13)a from the RBC to the bacteria was visualized.

Fresh RBC were pretreated with Rho-K_4-S4(1-13)a and co-cultured as described above. Cells in the co-culture were separated as described above, and the bacterial fraction examined under the fluorescent microscope at 580nm. As shown in Figure 17B, bacteria were labeled. This indicated that during the co-culture period, Rho-K_4-S4(1-13)a transferred from the RBC membrane to the bacteria.

EXAMPLE 13

Inhibition of fungal growth

Another example of the principle of Affinity Driven Molecular Transfer (ADMT) is provided using an antifungal assay. Human RBCs were pretreated with either 10μM K_4-S4(1-16)a or 10μM rhodaminated K_4-S4(1-16)a for 15min. as described above. After being washed, the LRBCs were co-cultured with the yeast cells *Cryptococcus neoformance* in RPMI complete medium (3h, 37°C). After the incubation period, RBCs were separated from the fungal cells using a 5μm filter (Sartorius). Control experiments included the use of *C. neoformance* co-cultured with RBC that

were pre-treated with either K_4-S4(1-16)a-free saline or with the supernatant from the last wash of K_4-S4(1-16)a-treated RBC. Inhibition of proliferation of the yeast cells was assessed by examination under microscope.

Growth of *Cryptococcus neoformance* is inhibited by the co-culture with RBC pretreated with K_4-S4(1-16)a. Fungal proliferation is inhibited in the presence of LRBC (Figure 18B) but not in the presence of normal RBC (Figure 18A). In addition, observation of these cells under fluorescent microscopy at 580nm revealed that they had become labeled (Figure 18C and 18D) which demonstrated that RBC-bound K_4-S4(1-16)a is able to transfer from the LRBC to *C. neoformance* and inhibit cell proliferation.

EXAMPLE 14

Inhibition of protozoan growth

Human RBC were pretreated with 50μM K_4-S4(1-16)a for 15min. as described above, unbound peptide is washed away (total bound peptide - as determined by HPLC analysis of the supernatant – is 7±1 μg). LRBC were subjected to hemolysis by adding in water. After several washes the resulting erythrocyte 'ghosts' were suspended in culture medium (complete RPMI 1640) and added to cultures of *Leishmania major* promastigotes (10^6/ml). For control experiments, untreated ghosts were used. After 16h. incubation at 27°C, an aliquot (25μl) from each sample was counted, the rest was washed and recultured in fresh medium, then re-counted after overnight incubation.

Growth of *Leishmania major* promastigotes was inhibited by the co-culture with RBC ghosts pretreated with K_4-S4(1-16)a. Leishmanial proliferation is inhibited in the presence of treated ghosts but not in the presence of untreated ghosts, as shown in Figure 19. Moreover, the treated cultures contain mostly cell debris and only a few intact and motile promastigotes were observed. Upon washing and cultured *de novo* culture in treated 'ghost'-free medium, the few surviving promastigotes did not proliferate, which demonstrates that ghost-bound K_4-S4(1-16)a, transferred from the LRBC to the *Leishmania major* promastigotes, retains is protozoacidal activity.

EXAMPLE 15

Intracellular delivery of the NLS coupled to K_4-S4(1-13)a

An example of the principle of ADMT of a peptide acting as a lipophilic "hppk" molecule, i.e the peptide has another molecule attached to it, is provided using a nuclear localization signal (NLS) covalently coupled to K_4-S4(1-13)a (to form K_4-S4(1-13)a-NLS). The NLS is reported to be responsible for directing a variety of intracellular molecules from the cytoplasm to the nucleus by interacting with a specific receptor bound to the nuclear membrane [28]. The activity of the NLS, while attached to the peptide affected the activity of the K_4-S4(1-13)a after transfer from the red blood cell membrane to another cell.

Human RBC (25µl) were exposed to the rhodaminated polypeptides K_4-S4(1-13)a or K_4-S4(1-13)a-NLS in PBS (20µM, 15 min. RT). After incubation, cells were thoroughly washed (3x) in PBS by successive centrifugations (2 min., 200 x g) then the pellet is suspended in complete RPMI 1640 culture medium and added to cultures of HeLa cells in LabTek culture chambers (50,000 cells/well cultured ON at 37°C, 5% CO_2). After the incubation period, RBC were washed away and cells were fixed and analyzed by Fluorescence Confocal Microscopy.

Confocal microscopic examination of HeLa cells from co-cultures in which the LRBC were pretreated with rhodaminated K_4-S4(1-13)a, showed that only the cytoplasm (not the nucleus) of HeLa cells is labeled (Figure 20A). In co-cultures pretreated with rhodaminated K_4-S4(1-13)a-NLS, both the cytoplasm and the nucleus of HeLa cells were labeled (Figure 20B). This demonstrated that RBC-bound K_4-S4(1-13)a or RBC-bound K_4-S4(1-13)a-NLS were able to transfer from the LRBC to the HeLa cells and reach the cytoplasm and/or the nucleus, respectively.

EXAMPLE 16

Resistance of LRBC to infection

An example for a potential prophylactic use of the principle of hooking dermaseptin peptides to RBC is provided, using cultures of human RBC that are infected by the malaria parasite *Plasmodium falciparum*. The infection rate of these

cultures is initially >95%, and the parasites are nearly all at the schyzont stage (ready to burst out of the host cell). LRBC that were pretreated with K_4-S4(1-13)a (as described above) were mixed with the infected cultures (cell ratio of 1:1) so that the infection rate at the onset of the co-culture is 50%. For control experiments, untreated RBC were used. After a 24h. incubation time of the mixed culture, the new infection rate is determined by counting the number of infected cells on an aliquot (50µl smear, Giemsa stained) from each sample.

RBC pretreated with K_4-S4(1-13)a are resistant to infection by *P. falciparum*. As shown in Figure 21, infection rate is increased in the presence of normal (untreated) RBC. In the presence of LRBC however, the infection rate is much lower. Observation under microscope revealed that the infected cells in all cultures were mostly at the ring stage (i.e., the first stage post infection), indicating *de novo* infection. This demonstrated that RBC with the antimicrobial peptide K_4-S4(1-13)a bound to the membrane were significantly less prone to infection by *P. falciparum*, than those without.

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The disclosures of all publications mentioned in the specification and of the publications cited therein are hereby incorporated by reference.

It is appreciated that this invention will most often be practiced with large numbers of red blood cells, of lipophilic hook molecules and of drug molecules. Therefore within the context of this application, where for clarity of description there is a singular usage of red blood cell, of lipophilic hook molecule and of drug molecule, it should be understood as referring to at least one red blood cell, at least one lipophilic hook molecule and at least one drug molecule, wherein "at least one" may refer to very large numbers.

It is appreciated that the particular embodiments described herein are intended only to provide a detailed disclosure of the present invention and are not intended to be limiting.

It is appreciated that various features of the invention which are, for clarity, described in the contexts of separate embodiments may also be provided in combination in a single embodiment. Conversely, various features of the invention which are, for brevity, described in the context of a single embodiment may also be provided separately or in any suitable subcombination.

It will be appreciated by persons skilled in the art that the present invention is not limited to what has been particularly shown and described hereinabove. Rather, the scope of the present invention is defined only by the claims that follow:

CLAIMS:

What is claimed is:

1. An amphipathic dermaseptin S4 derivative peptide selected from the group consisting of Seq. Id Nos. 2-19, and the sequence X-ALWKTLLKKVLKA-Y, where X and Y can be amine, tetramethyl-rhodamine, lissamine-rhodamine, lysine, an amino acid, Fmoc, Boc, a hydrophobic molecule or atom, said peptide having an antimicrobial activity and low hemolytic activity, an analog thereof, and pharmaceutically acceptable salts thereof.

2. A peptide of claim 1 wherein said hemolytic activity has an LC_{50} greater than about 10μM.

3. A peptide of claim 1 with substantially no hemolytic activity.

4. A nucleic acid sequence encoding a peptide of claim 1.

5. A peptide of claim 1 wherein said antimicrobial activity is a broad range antimicrobial activity.

6. A peptide according to claim 4 wherein said broad range antimicrobial activity comprises at least one selected from the group consisting of antiviral, antibacterial, antiprotozoan, antimycoplasma and antifungal activity.

7. A peptide of claim 6 wherein said antimicrobial activity is a prophylactic antimicrobial activity.

8. A peptide of claim 1 which has a low hydropathic index and a high positive charge.

9. A peptide of claim 8 wherein said low hydropathic index is less than about 10 and wherein said high positive charge is greater than about 4.

10. A peptide according to claim 1 which is in a substantially non-aggregated state in aqueous solution.

11. A peptide of claim 1 and also comprising a drug molecule attached to said peptide.

12. A peptide of claim 1 and also comprising a prophylactic molecule attached to

said peptide.

13. A peptide according to claim 11 wherein said drug molecule comprises an antimicrobial or an anticancer drug.

14. A peptide according to claim 1, which is effective to activate cells of the monocyte or macrophage lineage or other lymphoid cells.

15. At least one peptide of claim 1 suitable for stable attachment to the membrane of a red blood cell (RBC).

16. A peptide according to claim 15 wherein said stable attachment does not substantially affect said antimicrobial activity

17. A peptide according to claim 15, wherein said attachment is an affinity-driven attachment.

18. A peptide of claim 16, wherein said peptide is suitable for release from said RBC membrane in an affinity-driven release at a region of higher affinity for said peptide than the affinity of said peptide for said RBC membrane.

19. A peptide according to claim 18 wherein said region of higher affinity comprises a microbial pathogen.

20. A peptide according claim 19, wherein said microbial pathogen is selected from the group consisting of viral, bacterial, protozoan, mycoplasma and fungal pathogens.

21. A peptide according to claim 11 wherein said drug molecule is selected from the group consisting of oxacillin, vancomycin, ceftazidime and imipenem.

22. A peptide according to claim 18 wherein said region of higher affinity comprises a cancer or a tumor.

23. A peptide according to claim 11 wherein said drug molecule is selected from the group consisting of cyclophosphamide, methotrexate, 5-fluorouracil and loeucoverin.

24. A peptide according to claim 15 wherein said attachment occurs exteriorly to the body.

25. A peptide according to claim 15 wherein said attachment occurs within the bloodstream of a body.

26. A drug delivery method comprising,

a affinity-driven attachment of at least one lipophilic "hook" molecule to the cell membrane of at least one red blood cell (RBC); and

b affinity-driven release of at least one of said at least one lipophilic "hook" molecules from the cell membrane of said at least one RBCs at a region of higher affinity for said lipophilic "hook" molecule than the affinity of said lipophilic "hook" molecule for the cell membrane of said at least one RBC.

27. A method according to claim 26, wherein said at least one lipophilic "hook" molecule is a drug molecule.

28. A method according to claim 26. wherein at least one drug molecule is attached to said at least one lipophilic "hook" molecule.

29. A method according to claim 26 wherein said at least one lipophilic "hook" molecule is an amphipathic dermaseptin S4 derivative peptide selected from the group consisting of Seq. Id Nos. 2-19, and the sequence X-ALWKTLLKKVLKA-Y, where X and Y can be amine, tetramethyl-rhodamine, lissamine-rhodamine, lysine, an amino acid. Fmoc, Boc, a hydrophobic molecule or atom, said peptide having an antimicrobial activity and low hemolytic activity, an analog thereof, and pharmaceutically acceptable salts thereof.

30. A method according to claim 26, wherein said affinity-driven attachment of step a occurs exteriorly to a body.

31. A method according to claim 30, wherein after said attachment step a, and before step b. said at least one RBCs are introduced into the blood circulation of an individual.

32. A method according to claim 26, wherein said affinity-driven attachment of step a occurs within the bloodstream of a body.

33. A method according to claim 26, wherein said region of higher affinity comprises (the site of) a microbial pathogen, and wherein said drug molecule has an anti-microbial activity.

34. A method according to claim 33 wherein said microbial pathogen is selected

from the group consisting of viral, bacterial, protozoan and fungal pathogens.

35. A method according to claim 34 wherein the drug molecule is oxacillin, vancomycin, ceftazidime or imipenem.

36. A method according to claim 26 wherein said region of higher affinity comprises (the site of) a cancer/tumor, and wherein the drug molecule has an anti-cancer/tumor activity.

37. A method according to claim 36, wherein said cancer/tumor is a solid tumor or leukemia.

38. A drug delivery method comprising:

a. attachment of at least one lipophilic "hook" molecule to at least one drug molecule;

b. affinity-driven attachment of at least one of said at least one lipophilic "hook" molecules to the cell membrane of at least one RBC; and

c. affinity release of at least one of said lipophilic "hook" molecules from at least one of said at least one RBCs, at a region of higher affinity for the lipophilic "hook" molecule than the affinity of the lipophilic "hook" molecule for the RBC,

wherein steps a and b can be performed sequentially in either order, or substantially simultaneously.

39. A method according to claim 38 wherein said attachment steps a and b, occur exteriorly to a body.

40. A method according to claim 38 wherein, after said attachment steps a and b, and before step c, said at least one RBCs are introduced into the blood circulation of an individual.

41. A method according to claim 38, wherein said affinity-driven attachment of step b occurs within the blood circulation of a body.

42. A kit for the attachment of at least one lipophilic "hook" molecule to at least one red blood cell (RBC), the kit comprising said at least one lipophilic "hook" molecule, and reagents suitable for attachment of said at least one lipophilic "hook" molecule to the cell membrane of said at least one RBC.

43. A kit according to claim 42 wherein said lipophilic "hook" molecule comprises a drug molecule.

44. A kit for the attachment of at least one drug molecule to at least one red blood cell (RBC), the kit comprising:

at least one lipophilic "hook" molecule;

reagents suitable for attachment of said at least one drug molecule to said at least one lipophilic "hook" molecule; and

reagents suitable for attachment of said at least one lipophilic "hook" molecule to the cell membrane of at least one RBC.

45. A kit according to claim 44 and also comprising said at least one drug molecule.

46. A kit according to claim 44 and also comprising apparatus suitable for separating the RBCs with said at least one lipophilic "hook" molecule attached, from the other reagents.

47 A kit according to claim 44 and also comprising RBCs in a form suitable for the attachment of said at least one lipophilic "hook" molecule.

48. A kit according to claim 44 and also comprising instructions for the proper usage of said kit.

49. A method of introducing a molecule into the interior of a mammalian cell not including a RBC, the method comprising

a. attachment of said molecule to an amphipathic dermaseptin derivative peptide of claim 1; and

b. co-incubating said cationic amphipathic dermaseptin derivative peptide in the presence of said at least one mammalian cell, whereby said molecule attached to said cationic amphipathic dermaseptin derivative peptide enters the cell.

50. A drug delivery method comprising

a affinity-driven attachment of a peptide to a red blood cell (RBC) membrane; and

b affinity-driven release of said peptide from the RBC membrane at a

region of higher affinity for said peptide than the affinity of said peptide for the RBC membrane,

wherein said peptide is a drug.

51. A drug delivery method comprising:

a. attachment of a plurality of drug molecules to a plurality of peptides;

b. affinity-driven attachment of at least some of said plurality of peptides to a plurality of RBC membranes; and

c. affinity-driven release of at least some of said peptides from at least some of said RBCs at a region of higher affinity for said peptide than the affinity of said peptide for said RBC membrane,

wherein steps a and b can be performed sequentially in either order, or substantially simultaneously.

52. A method of attachment of at least one drug molecule to at least one RBC membrane, the method comprising co-incubating at least one peptide of claim 1 in the presence of at least one RBC, whereby said at least one peptide attaches to said RBC membrane in an affinity-driven attachment.

53. A method according to claim 52 wherein said peptide is a drug molecule.

54. A method according to claim 52 wherein at least one drug molecule is attached to said at least one peptide.

55. A method according to claim 52, wherein said affinity-driven attachment occurs within the blood circulation of a body.

56. A method according to claim 52, wherein said affinity-driven attachment occurs exteriorly to a body.

57. A method according to claim 56, wherein said at least one RBC, with said at least one peptide attached to the cell membrane, is introduced to the blood circulation of the body.

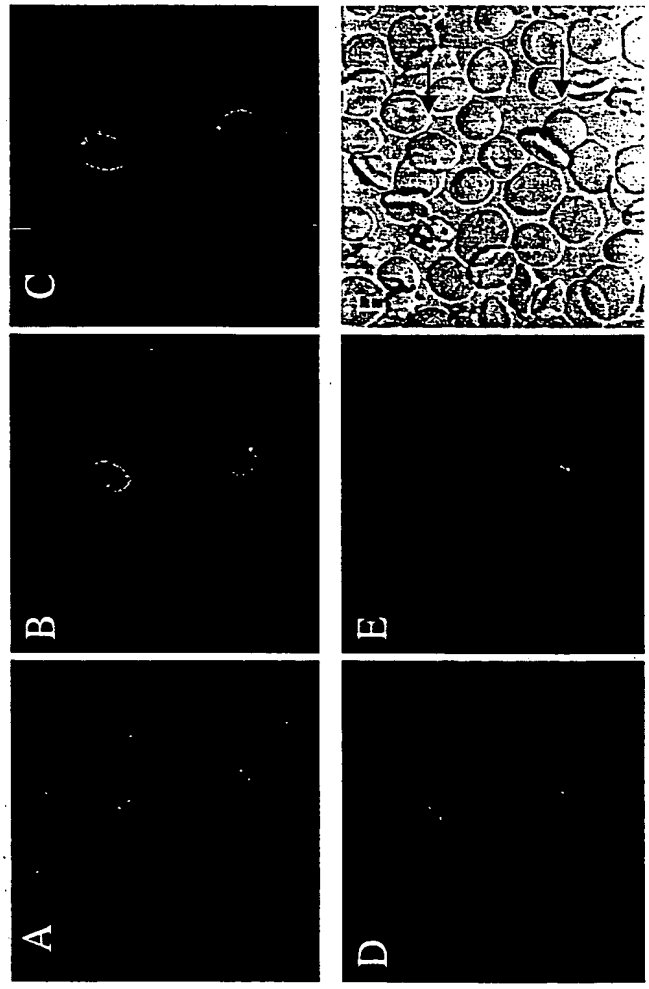


Figure 1

Figure 2

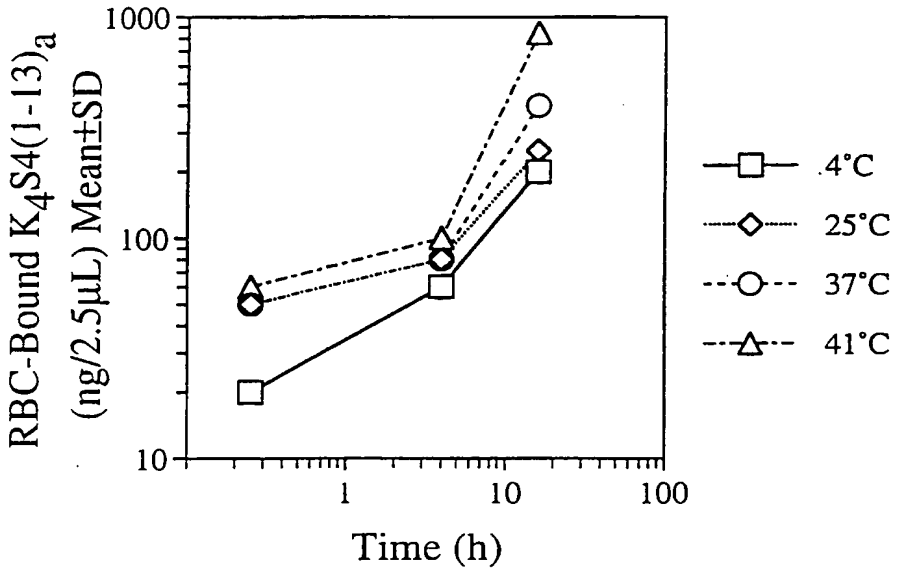

RBC-Bound $K_4S4(1-13)_a$
(ng/2.5µL) Mean±SD
1000
100
10
1
10
100
Time (h)
4°C
25°C
37°C
41°C

Figure 3

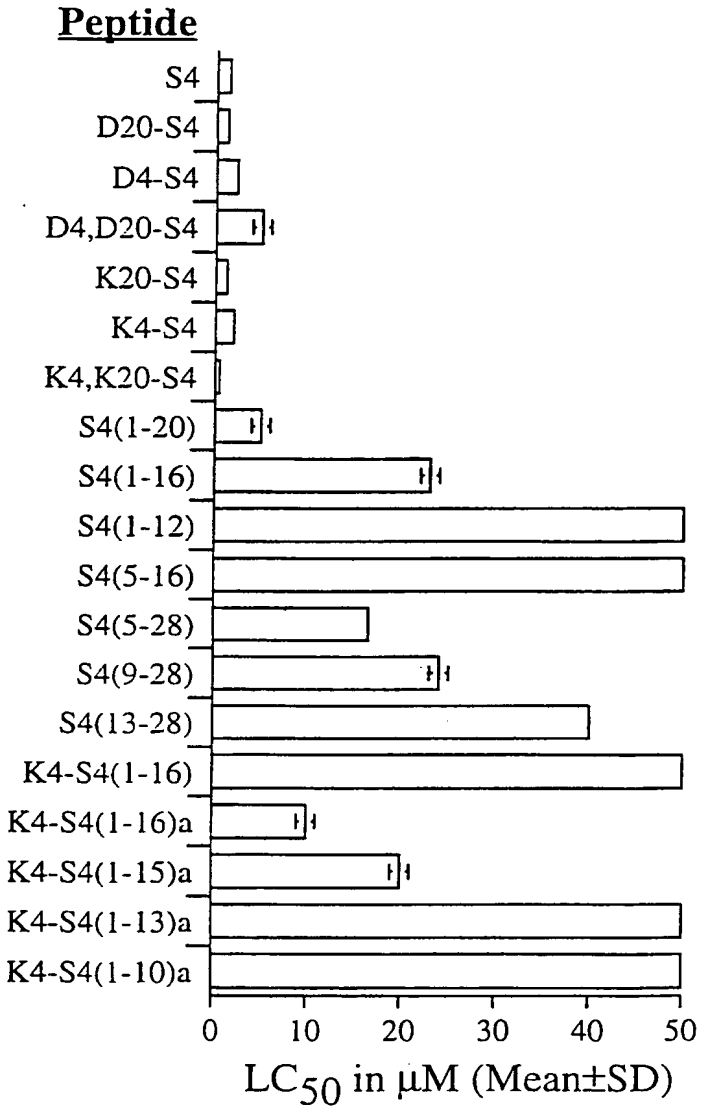

Peptide
S4
D20-S4
D4-S4
D4,D20-S4
K20-S4
K4-S4
K4,K20-S4
S4(1-20)
S4(1-16)
S4(1-12)
S4(5-16)
S4(5-28)
S4(9-28)
S4(13-28)
K4-S4(1-16)
K4-S4(1-16)a
K4-S4(1-15)a
K4-S4(1-13)a
K4-S4(1-10)a
0
10
20
30
40
50
LC50 in µM (Mean±SD)

Figure 4

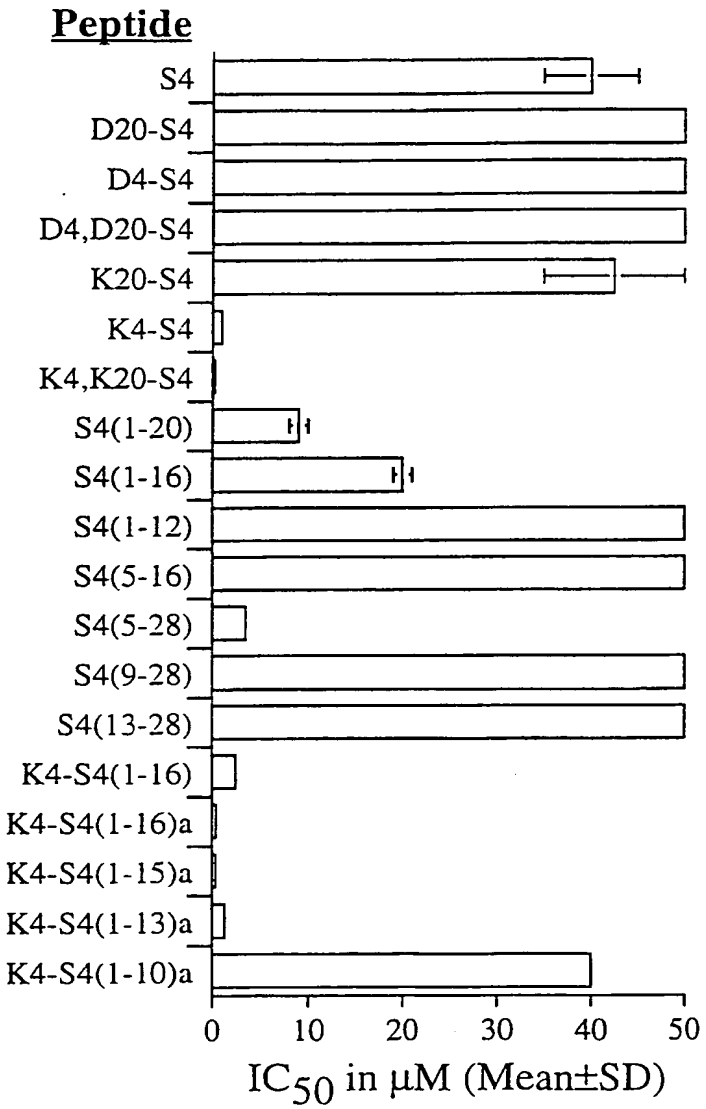

Peptide
S4
D20-S4
D4-S4
D4,D20-S4
K20-S4
K4-S4
K4,K20-S4
S4(1-20)
S4(1-16)
S4(1-12)
S4(5-16)
S4(5-28)
S4(9-28)
S4(13-28)
K4-S4(1-16)
K4-S4(1-16)a
K4-S4(1-15)a
K4-S4(1-13)a
K4-S4(1-10)a
0
10
20
30
40
50
IC50 in µM (Mean±SD)

Figure 5

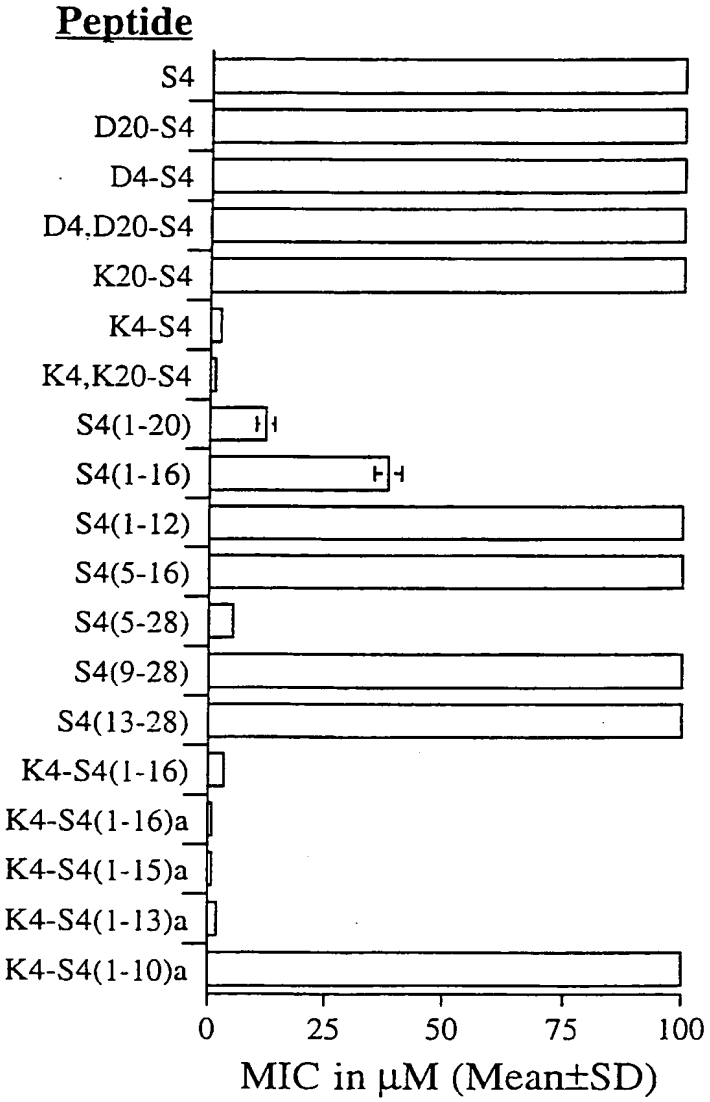

Peptide
S4
D20-S4
D4-S4
D4,D20-S4
K20-S4
K4-S4
K4,K20-S4
S4(1-20)
S4(1-16)
S4(1-12)
S4(5-16)
S4(5-28)
S4(9-28)
S4(13-28)
K4-S4(1-16)
K4-S4(1-16)a
K4-S4(1-15)a
K4-S4(1-13)a
K4-S4(1-10)a
0
25
50
75
100
MIC in μM (Mean±SD)

Figure 6

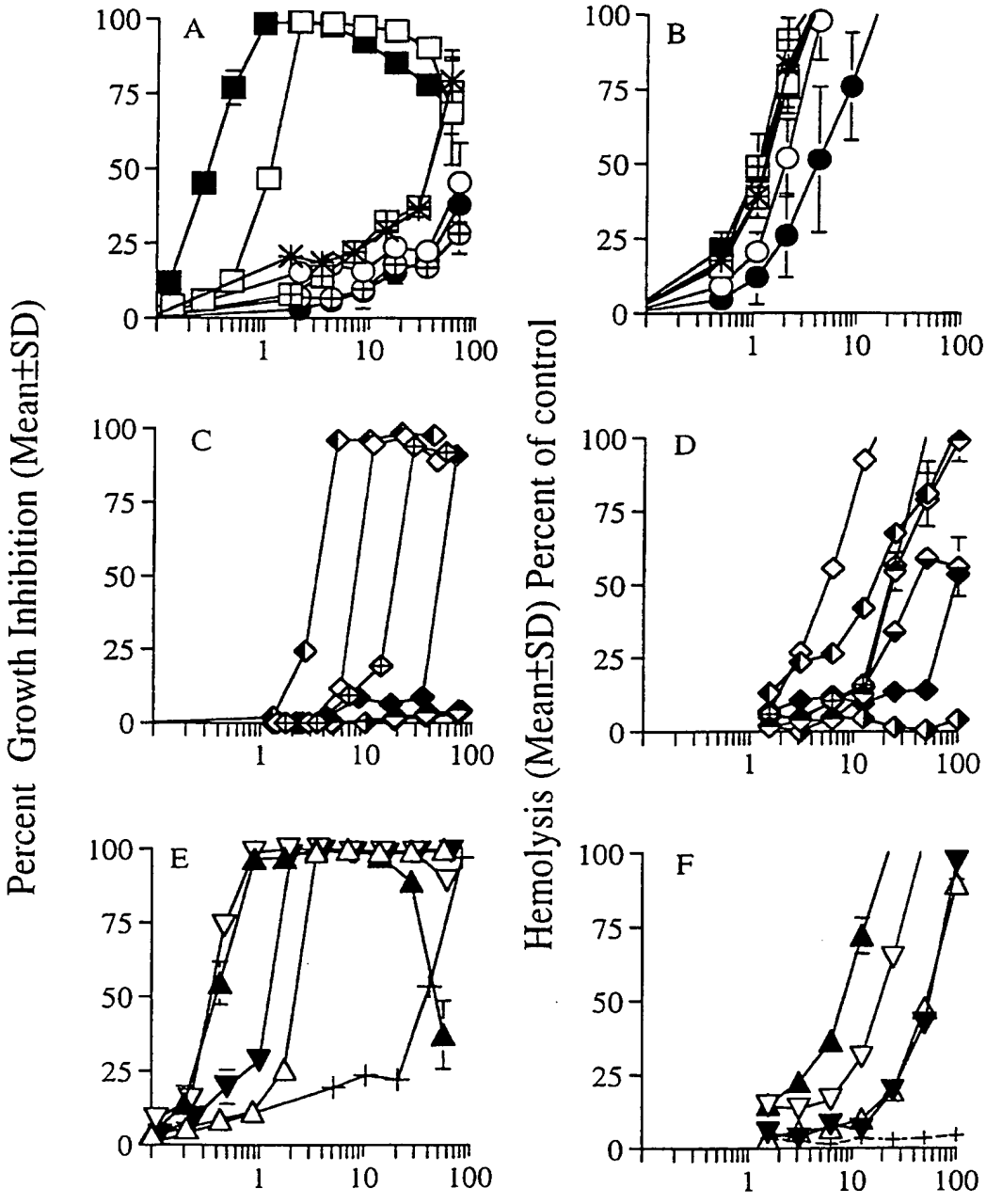

A
B
C
D
E
F
100
75
50
25
0
1
10
100
Percent Growth Inhibition (Mean±SD)
Hemolysis (Mean±SD) Percent of control
Peptide Concentration (μM)

Figure 7

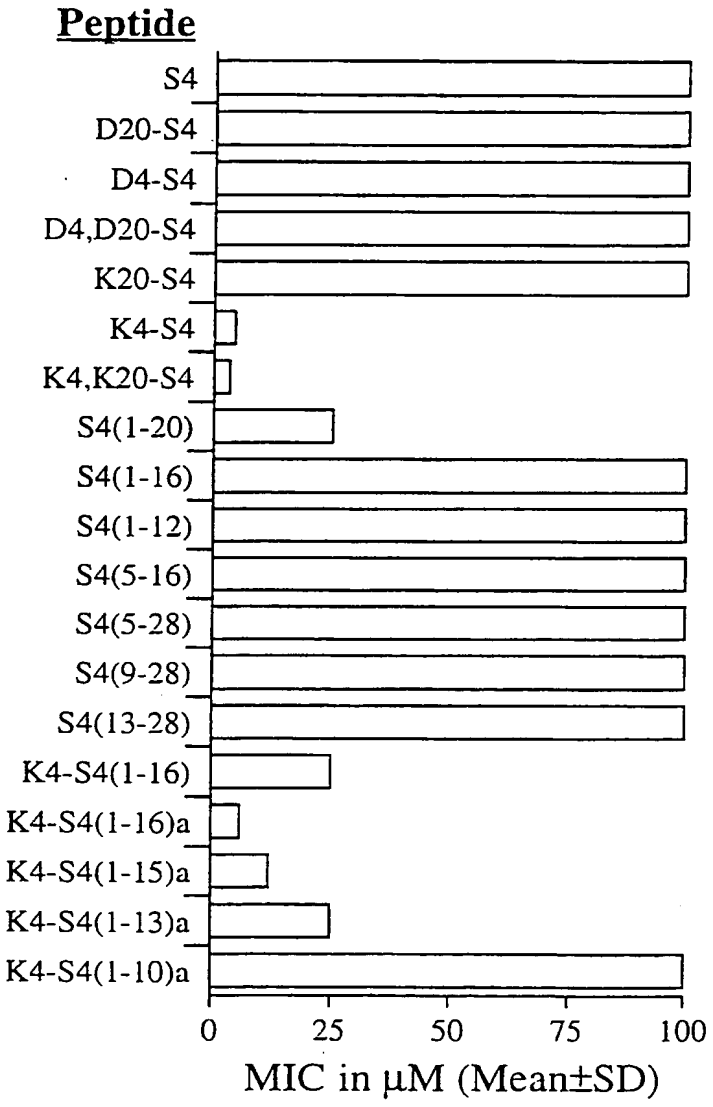

Peptide
S4
D20-S4
D4-S4
D4,D20-S4
K20-S4
K4-S4
K4,K20-S4
S4(1-20)
S4(1-16)
S4(1-12)
S4(5-16)
S4(5-28)
S4(9-28)
S4(13-28)
K4-S4(1-16)
K4-S4(1-16)a
K4-S4(1-15)a
K4-S4(1-13)a
K4-S4(1-10)a
0
25
50
75
100
MIC in μM (Mean±SD)

Figure 8

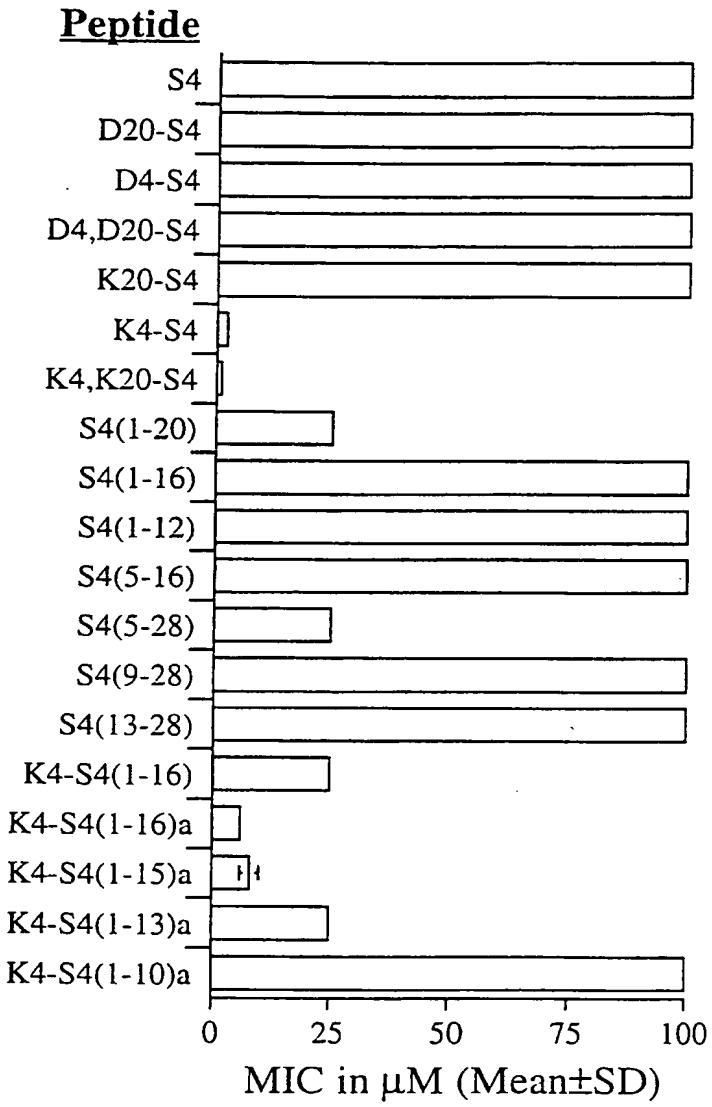

Peptide
S4
D20-S4
D4-S4
D4,D20-S4
K20-S4
K4-S4
K4,K20-S4
S4(1-20)
S4(1-16)
S4(1-12)
S4(5-16)
S4(5-28)
S4(9-28)
S4(13-28)
K4-S4(1-16)
K4-S4(1-16)a
K4-S4(1-15)a
K4-S4(1-13)a
K4-S4(1-10)a
0
25
50
75
100
MIC in μM (Mean±SD)

Figure 9

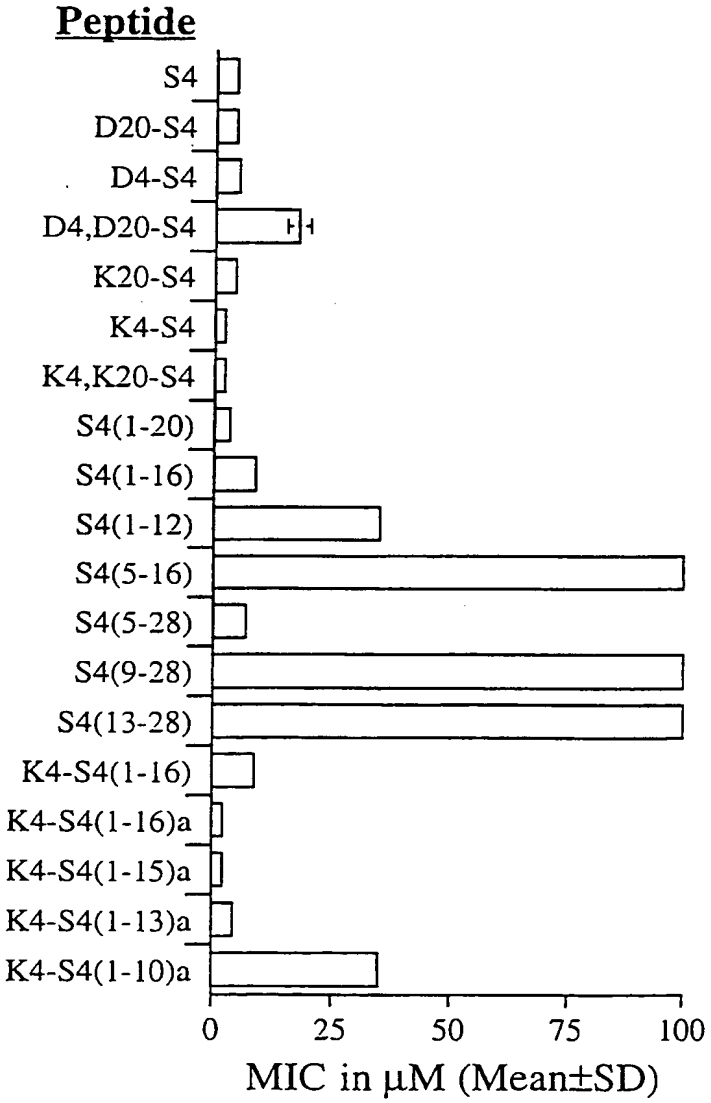

Peptide
S4
D20-S4
D4-S4
D4,D20-S4
K20-S4
K4-S4
K4,K20-S4
S4(1-20)
S4(1-16)
S4(1-12)
S4(5-16)
S4(5-28)
S4(9-28)
S4(13-28)
K4-S4(1-16)
K4-S4(1-16)a
K4-S4(1-15)a
K4-S4(1-13)a
K4-S4(1-10)a
0
25
50
75
100
MIC in μM (Mean±SD)

Figure 10

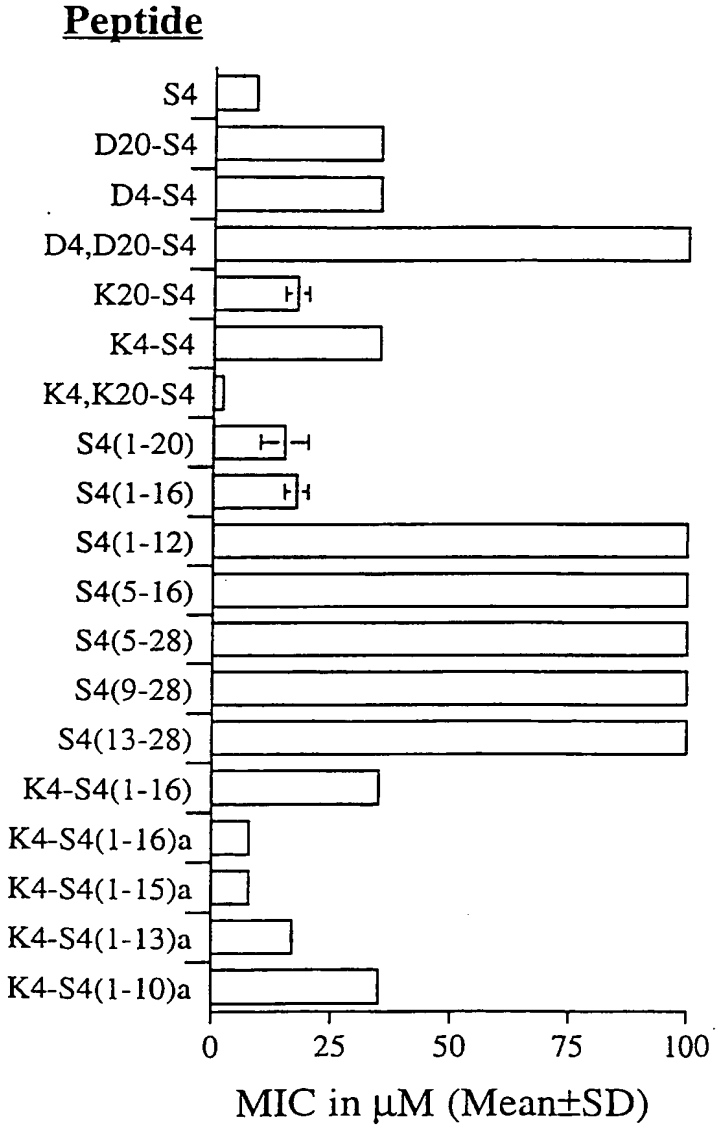

Peptide
S4
D20-S4
D4-S4
D4,D20-S4
K20-S4
K4-S4
K4,K20-S4
S4(1-20)
S4(1-16)
S4(1-12)
S4(5-16)
S4(5-28)
S4(9-28)
S4(13-28)
K4-S4(1-16)
K4-S4(1-16)a
K4-S4(1-15)a
K4-S4(1-13)a
K4-S4(1-10)a
0
25
50
75
100
MIC in μM (Mean±SD)

Figure 11

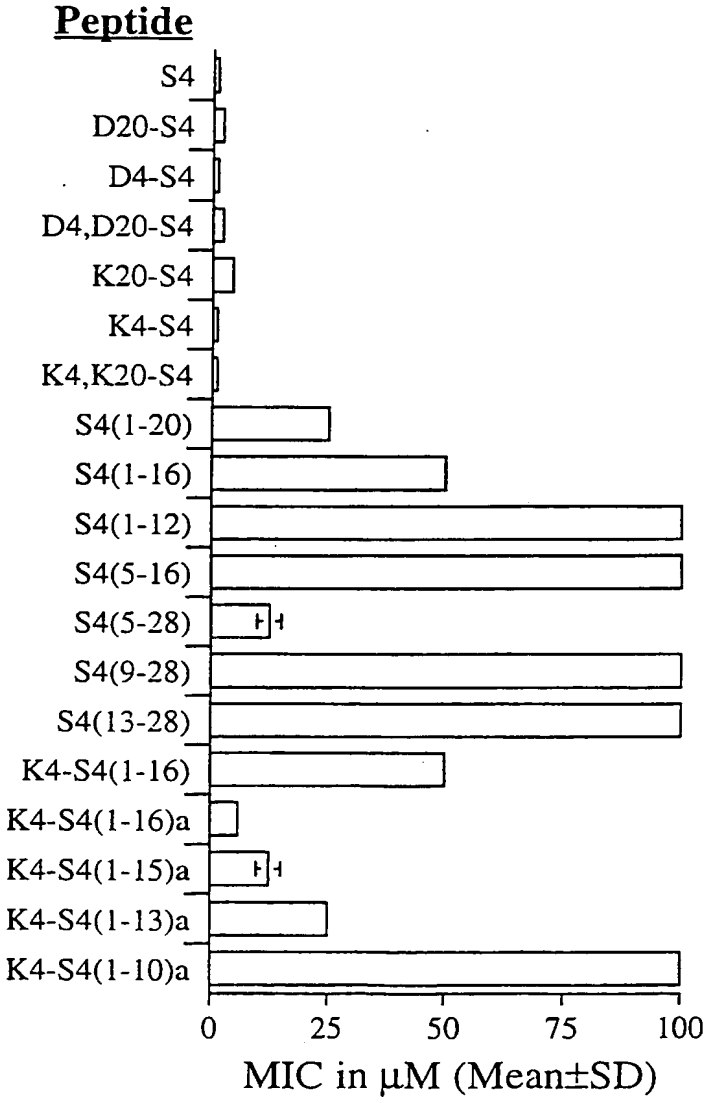
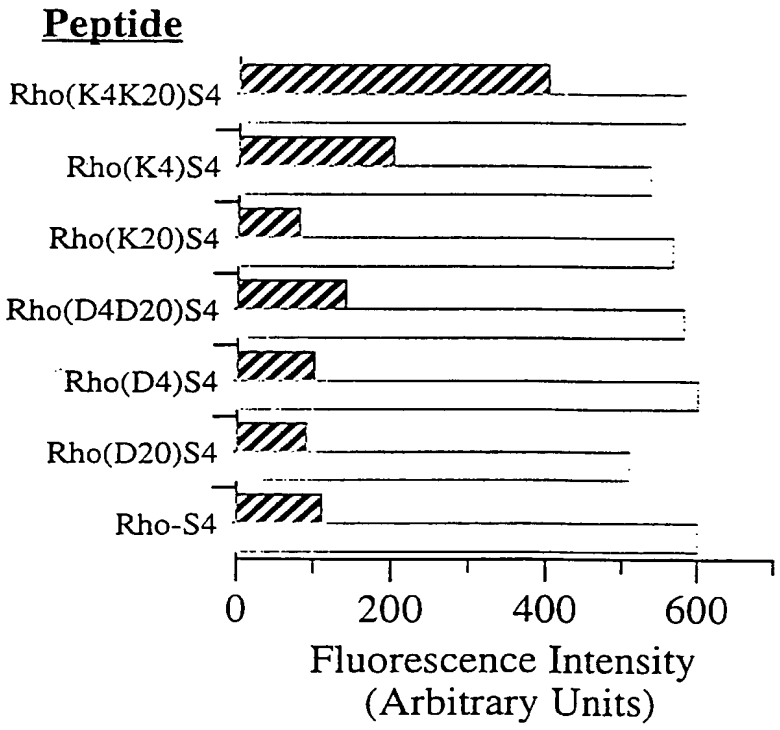

Peptide
S4
D20-S4
D4-S4
D4,D20-S4
K20-S4
K4-S4
K4,K20-S4
S4(1-20)
S4(1-16)
S4(1-12)
S4(5-16)
S4(5-28)
S4(9-28)
S4(13-28)
K4-S4(1-16)
K4-S4(1-16)a
K4-S4(1-15)a
K4-S4(1-13)a
K4-S4(1-10)a
0
25
50
75
100
MIC in μM (Mean±SD)

Figure 12


Peptide
Rho(K4K20)S4
Rho(K4)S4
Rho(K20)S4
Rho(D4D20)S4
Rho(D4)S4
Rho(D20)S4
Rho-S4
0
200
400
600
Fluorescence Intensity
(Arbitrary Units)

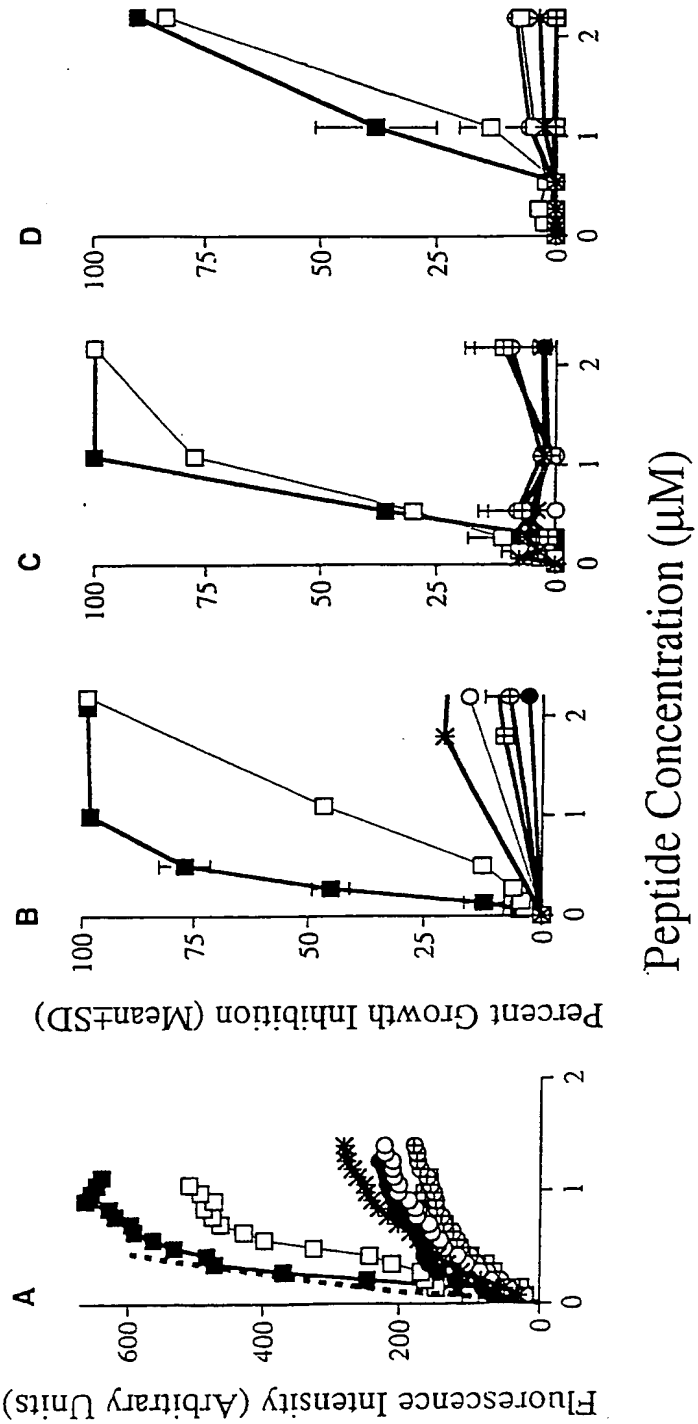

A
B
C
D
Fluorescence Intensity (Arbitrary Units)
Percent Growth Inhibition (Mean±SD)
Peptide Concentration (μM)
0
200
400
600
25
50
75
100
1
2

Figure 13

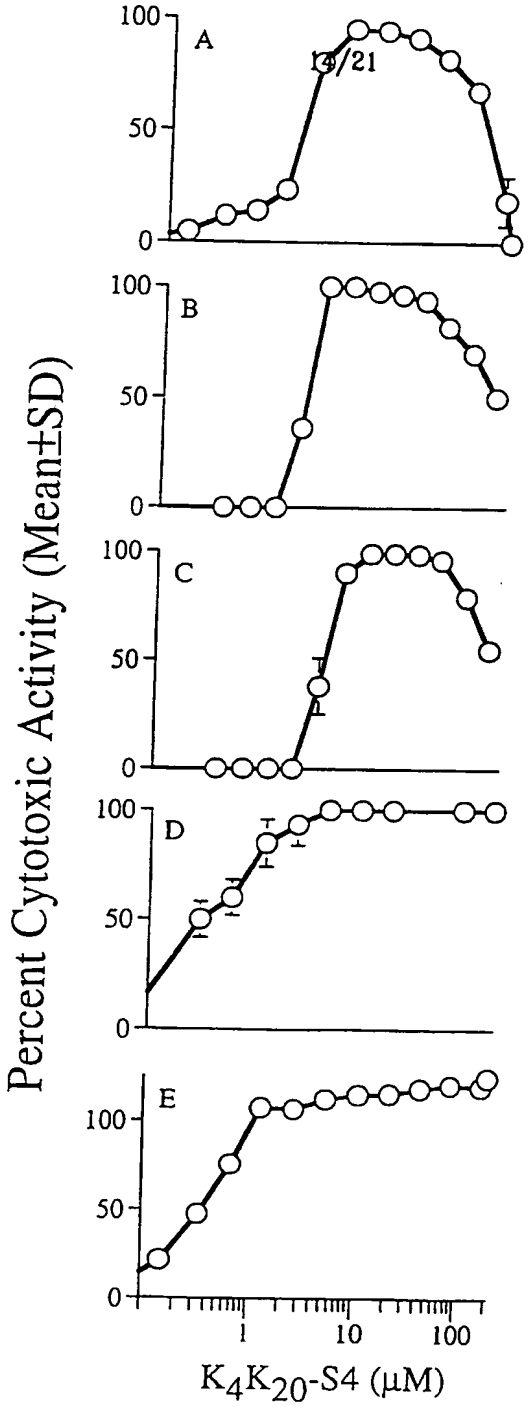

Percent Cytotoxic Activity (Mean±SD)
A
B
C
D
E
100
50
0
1
10
100
K_4K_{20}-S4 (μM)

Figure 14

Figure 15

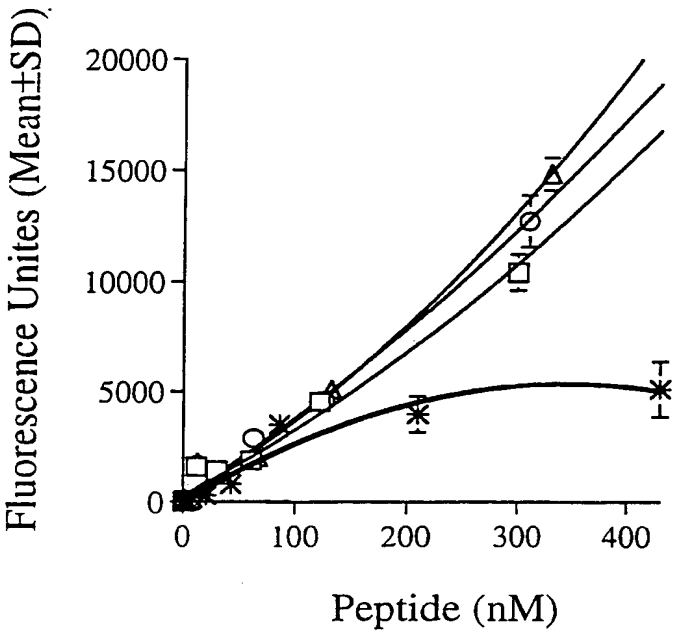
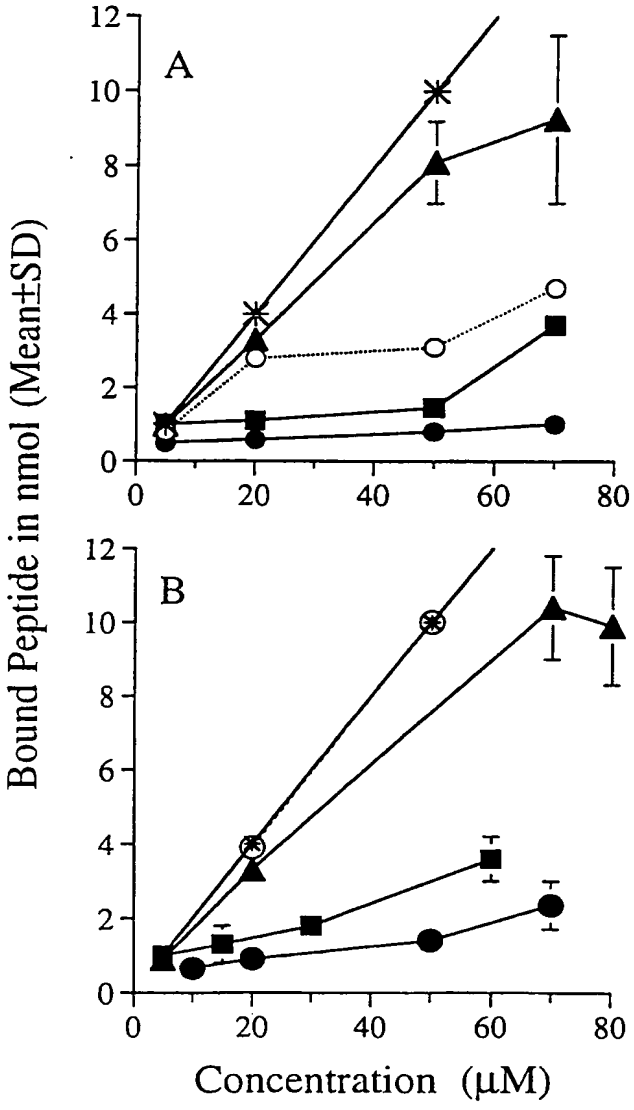
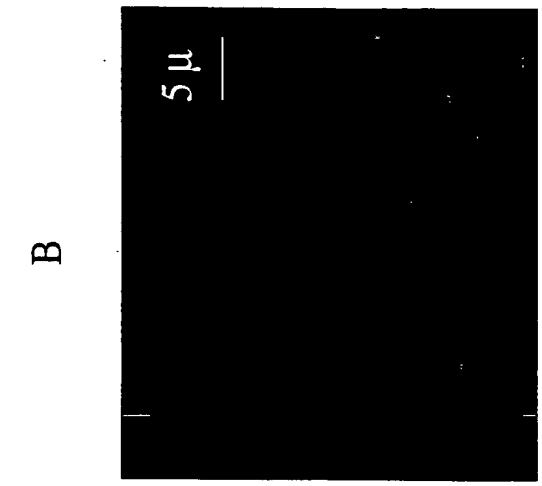
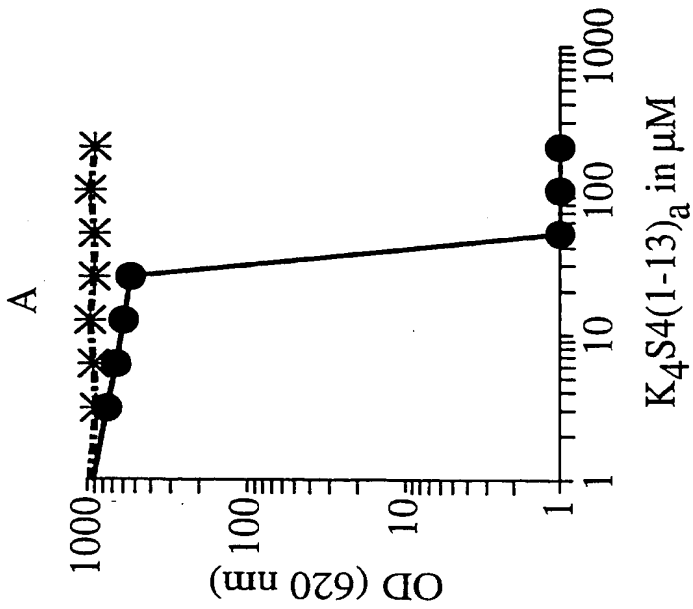

Fluorescence Unites (Mean±SD)
20000
15000
10000
5000
0
0
100
200
300
400
Peptide (nM)

Figure 16


A
B
Bound Peptide in nmol (Mean±SD)
Concentration (μM)
0
2
4
6
8
10
12
0
20
40
60
80





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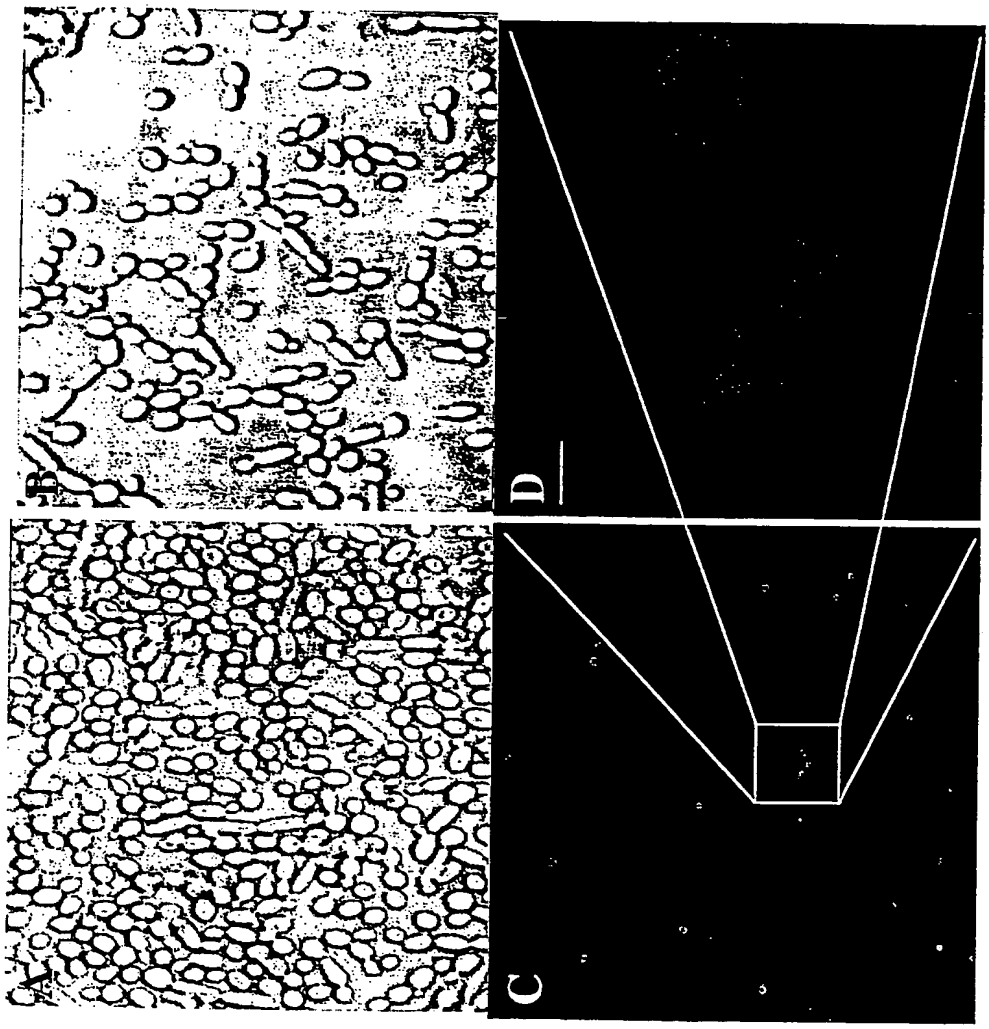
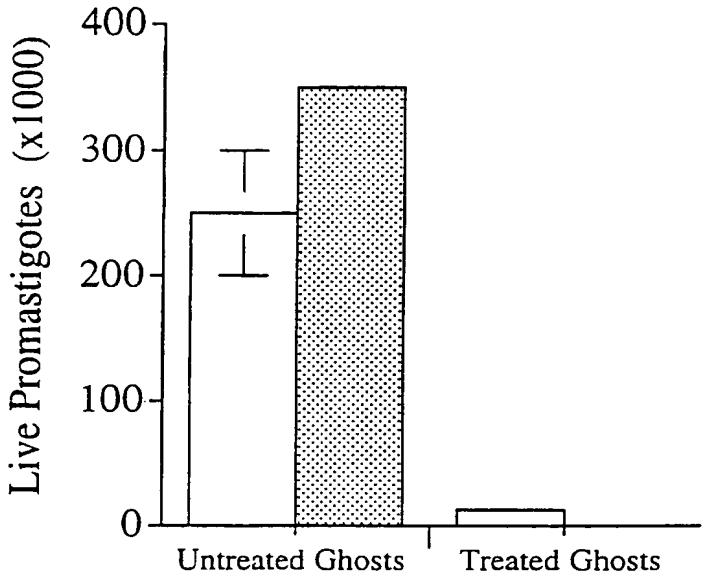


Figure 18

Figure 19


400
300
200
100
0
Live Promastigotes (x1000)
Untreated Ghosts
Treated Ghosts

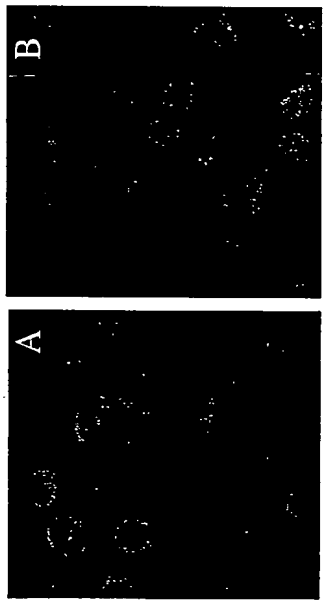
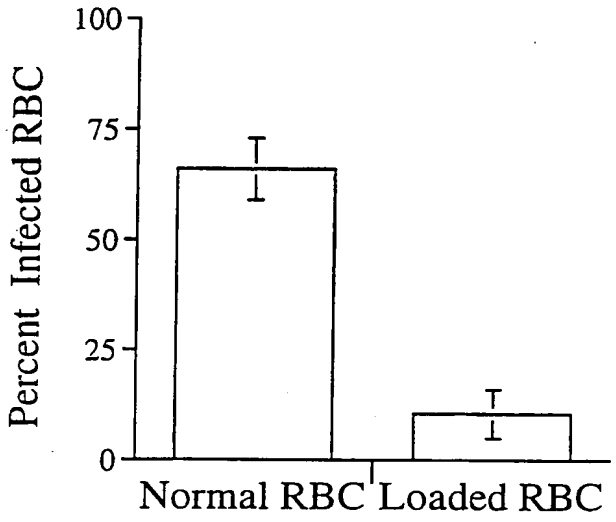


Figure 20

Figure 21



SEQUENCE LISTING

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Nicolas, Pierre
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from Frog Skin
<303> Eur. J. Biochem.
<304> 219
<306> 145-154
<307> Feb 1994

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20 25

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20 25

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20

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1 5 10 15

<210> 18
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(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: **MOR, Amram** [IL/IL]; Yeffe Nof Street 12/3, 96183 Jerusalem (IL).

(74) Agents: **COLB, Sanford, T.** et al.; Sanford T. Colb & Co., P.O. Box 2273, 76122 Rehovot (IL).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

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Published:
— *With international search report.*

(88) Date of publication of the international search report:
5 July 2001

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/10887 A3

(54) Title: MULTI-FUNCTIONAL ANTIMICROBIAL PEPTIDES

(57) Abstract: This application discloses novel dermaseptin derived peptides with a broad range antimicrobial activity and a reduced hemolytic activity. The affinity of the peptides for the plasma membrane of red blood cells (RBCs), allows the use of RBCs as a drug delivery vehicle.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL00/00482

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :A61K 38/10; C07K 7/08
US CL : 514/13,14; 530/326,327
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/13,14; 530/326,327

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN on line

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	AMMAR, B. et al. Dermaseptin, a Peptide Antibiotic, Stimulates Microbicidal Activities of Polymorphonuclear Leukocytes. Biochim. Biophys. Res. Commun. 29 June 1998, Vol. 247, pages 870-875, see entire document.	1-3, 5-10, 14-20, 22, 24,25
X	WO 96/28468 A2 (UNILEVER N.V.) 19 September 1996, see claim 2.	1-3, 5-10, 14-20, 22, 24,25
X	NICOLAS, P. et al. Peptides as weapons against microorganisms in the chemical defense system of vertebrates. Annu. Rev. Microbiol. 1995, Vol. 49, pages 277-304, specifically page 285.	1-3, 5-10, 14-20, 22, 24,25

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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Date of the actual completion of the international search 16 JANUARY 2001	Date of mailing of the international search report 29 JAN 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Julie Burke Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL00/00482

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-3 and 5-25

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL00/00482

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional examination fees must be paid.

Group I, claims 1-3,5-25, drawn to peptide product.
Group II, claim 4,drawn to nucleic acid encoding product of Group I.
Group III, claims 26-28,38-41,50,51 drawn to generic drug delivery method and claims 42-48, drawn to the kit to be used therein.
Group IV, claims 29-37, 52-57, drawn to method of use of product of Group I.
Group V, claim 49, drawn to method of introducing a molecule into interior of a cell.

The inventions listed as Groups I-V do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Inventions of Groups I and II are structurally and functionally different products which are made by different methods and have different uses. Inventions I and II are separate and distinct because the inventions are directed to different chemical types regarding the critical limitations therein. For Group II, the critical feature is a polynucleotide whereas for Group I the critical feature is a polypeptide. It is acknowledged that various processing steps may cause a polypeptide of group I to be directed as to its synthesis by a polynucleotide of Group II, however, the completely separate chemical types of the inventions of Groups I and II supports the undue search burden if both were examined together. Additionally, polypeptides have been most commonly, albeit nor always, separately characterized and published in the Biochemical literature, thus significantly adding to the search burden if examiner together, as compared to being searched separately. Also, it is pointed out that processing that may connect two groups does not prevent them from being viewed as distinct, because enough processing can result in producing any composition from any other composition if the processing is not so limited to additions, subtractions, enzyme actions, etc.

For Groups I and IV/V Group I is the technical feature that links Groups I to Groups IV or V. Group I is not the contribution over the prior art because it is suggested by references teaching analogs of peptides of claim 1. For example, such peptide analogs are taught in WO 9628468,Nicolas et al (Annu. Rev. Microbiol., 49, 277-304, 1995; reference AL in the parent US case), Ammar et al (Biochim. Biophys. Res. Commun., 1998, Jun 29, 247: 870-875). Therefore, the lack of unity is present because the linking technical feature is not a "special technical feature" as defined by PCT Rule 13.2.

Groups I and III are distinct as they do not have common technical feature linking them. The scope of Group III is different as it does not recite products of Group I.

Groups III and V are distinct as they have different functions and method of Group III does not require the step of co-incubating with dermaseptin peptide required for Group V.